

Role of 5 α -Reduced Androgens in the Ovary

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I am also extremely appreciative of my son Victor for putting up with long hours away from home and missed soccer matches.

Declaration

This thesis is wholly the result of my own work. No part of it has been submitted to any other board for another qualification and the views expressed are those of the author and not of the University.

Presentations

Tetsuka, M., Milne, M., Simpson, GE., Mubiru, JN., Hillier, SG., 1998. Down regulation of 5 α -reductase mRNA expression during the preovulatory period in rat ovary. *Journal of Endocrinology Supplements* (1998) 156:266

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ABBREVIATIONS

3 α -HSD	3 α -hydroxysteroid dehydrogenase
5 α -A	5 α -androstanedione
5 α -R1	5 α -reductase type 1
5 α -R2	5 α -reductase type 2
5 β -A	5 β -androstanedione
5 β -R	5 β -reductase
17 β -HSD	hydroxysteroid dehydrogenase
17KA	17ketoandrogens
18S	18 Sverdberg units ribosomal RNA
28S	28 Sverdberg units ribosomal RNA
Aromatase	cytochrome P450 _{arom} ; product of CYP19 gene
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CG	chorionic gonadotrophin
DAB	3,3-diaminobenzadine tetrahydrochloride
DCS	donor calf serum
DEPC	diethyl pyrocarbonate
DES	diethylstilboestrol
DHT	dihydrotestosterone
DNAse	deoxyribonuclease
dNTP	deoxyribonucleotide
DTT	dithiothreitol
eCG	equine chorionic gonadotrophin
EDTA	ethylenediaminetetraacetic acid
FSH	follicle stimulating hormone
hCG	human chorionic gonadotrophin
IAA	isoamyl alcohol
ICC	immunocytochemistry
IPTG	isopropyl beta-D-thiogalactoside
ISH	<i>in situ</i> hybridisation
KB	kilobases

LB medium	Luria Bertani medium
LH	lutetising hormone
mRNA	messenger ribonucleic acid
MMLV	Moloney Murine Leukemia Virus
NBF	normal buffered formaldehyde
NSB	non-specific binding
NSS	normal swine serum
PIPES	1,4-piperazinediethanesulfonic acid
PBS	phosphate buffered saline
PGBS	phosphate buffered saline containing gelatine
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PFA	paraformaldehyde
Rf value	mobility relative to the solvent front
RIA	radioimmunoassay
RNase	ribonuclease
RPA	RNase protection assay
Rnasin	RNase inhibitor
rRNA	ribosomal RNA
s.c	subcutaneously
SSC	sodium chloride and sodium citrate
TAE	Tris-acetate EDTA buffer
TBS	Tris-buffered saline
TE	Tris HCl-EDTA buffer
TIC	thecal interstitial cells

ABSTRACT

Ovarian androgens are products of steroidogenic processes that are integral to follicular development, which culminates in ovulation. Follicle development involves growth and differentiation of the different follicular cell types. These developments enable maturing follicles to become steroidogenically competent and eventually release mature oocytes capable of fertilisation. The follicular cells involved in steroidogenesis are granulosa (GC) and theca cells (TC). Androgens are synthesised in TC. The main ovarian androgens are androstenedione and testosterone, which are predominantly substrates for aromatisation by GC into oestrogens, the most physiologically important steroids in the female. Follicle stimulating hormone (FSH) and luteinising hormone (LH) regulation of steroid synthesis is well documented. It is now also known that locally produced regulators within the ovary modulate this endocrine control. Autocrine/paracrine control modulates gonadotrophin-induced ovarian proliferation, differentiation and steroidogenesis.

This thesis researches steroid 5α -androstenedione (5α -A), a product of 5α -reductive metabolism of androstenedione. 5α -A is an aromatase inhibitor which inhibits oestradiol production, and therefore has a potential intraovarian paracrine role in modulating oestrogen biosynthesis. I investigated whether 5α -A was a product of androstenedione metabolism in the rat ovary, as an animal model. Since 5α -A is a metabolic product of 5α -reductase type 1 (5α -R1), a steroidogenic enzyme present in human GC and TC, the thesis also describes studies of the localisation and gonadotrophic regulation of 5α -R1 in the rat ovary. Ovarian aromatase expression was also investigated because 5α -R1 activity is associated with inhibition of oestrogen production, which depends on aromatase activity.

The metabolism of androstenedione was investigated in *in vitro* cultures of isolated ovarian GC and TCs. The steroid metabolites were investigated using radiochromatography. In GC cultures, oestradiol was detected among the steroid metabolites, but 5α -A was not. Additional investigations using oestradiol radioimmunoassay (RIA) supported the findings. The lack of a method for measurement of 5α -A led to the development and validation of a new RIA for this

purpose. However, the assay crossreacted significantly with androsterone and androstenedione as well as 5 α -A. Although thin layer chromatography (TLC) could resolve the steroids, incorporation of the separation technique in the assay of biological samples was problematic due to inconsistent steroid recoveries. Therefore, the assay could only be used to measure total 17keto-androgens (17KAs) in unpurified biological samples.

The location of 5 α -R1 in the rat ovary was determined to identify the ovarian cell types responsible for 5 α -A production. 5 α -R1 messenger RNA (mRNA) and protein were detected in the theca/interstitial cells (TIC) of the rat ovary, and strongly expressed in immature ovaries. Although 5 α -R1 tissue expression pattern was similar, levels were markedly reduced in adult ovaries. Northern analysis and *in situ* hybridisation (ISH) clearly showed that 5 α -R1 mRNA was abundantly expressed in the TIC. Additional evidence by immunocytochemistry (ICC) depicted the same location of 5 α -R1 protein. 5 α -R1 was not localised in rat GC. The expression of 5 α -R1 in the immature rat ovary was developmentally regulated by gonadotrophins because eCG decreased its expression while it was transiently up-regulated by hCG. Because each hormone acts on either granulosa or theca cells in immature ovaries, hCG stimulation of 5 α -R1 expression suggested that LH acts on theca cells to stimulate 5 α -R1 expression. ECG down-regulated 5 α -R1 expression, suggesting that FSH induced this action by local agents produced in the granulosa cells. These factors are yet to be identified, but this action of FSH indicates a paracrine product of GC that inhibits 5 α -R1 expression. ECG and hCG induced developmental changes reflecting the follicular changes that occur before ovulation. The findings, therefore, suggest that FSH, (the secretion of which is known to increase early in follicle development) decreases ovarian 5 α -R1 expression. However, expression is stimulated by LH, which rises around the time of ovulation. Hence, 5 α -R1 action and 5 α -reduced androgen production are reduced when follicle development begins, probably to prevent an inhibitory action on oestrogen synthesis. The transient increase in 5 α -R1 around ovulation suggests a regulatory role of 5 α -reduced steroids at this stage of follicle development.

The location of 5 α -R1 in rat TIC indicated that they were the cells of choice for cell culture investigations of 5 α -R1 activity. However, repeated attempts to

demonstrate 5 α -R1 activity in TC in isolated cell cultures were unsuccessful, even though the cited literature reported 5 α -R1 activity in rat GC and TC. The results of aromatase investigations were similar to reported findings. Aromatase mRNA was expressed abundantly in the GC of eCG-treated ovaries, but absent in GC of immature or hCG-treated ovaries as well as the TIC. The findings also matched the well-known stimulatory role of FSH on aromatase action.

Finally, other investigators had implicated 5 α -A in dysfunctional follicle growth in women presenting with polycystic ovary syndrome (PCOS). Therefore, the concluding clinical section of the thesis is devoted to the measurement of 5 α -A levels in follicular fluids (FF) obtained from the ovaries of women with normal ovarian function and those with PCOS. Measurements of androstenedione and 5 α -A in normal and PCOS FF showed that both androgens were elevated in PCOS subjects, whether ovulatory or anovulatory, compared to normal FF. The findings illustrated abnormal steroid production in PCOS ovaries and are reflective of the characteristic hyperandrogenaemia of the condition.

In summary, this is the first definitive description of the spatiotemporal expression of 5 α -R1 in the mammalian ovary. 5 α -R1 gene expression was located to rat TIC and shown to be regulated developmentally by gonadotrophins. A new RIA was developed and used to measure the 5 α -reduced androgens and androstenedione in normal and PCOS FF. These androgens were shown to be elevated in PCOS, where they probably contribute to the local intraovarian paracrine control that impairs oestradiol production in anovulatory PCOS.

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Ovarian androgen secretion begins in vivo (Lewy et al., 1979; Jostad, 1982) before the production of female sex steroids, which regulate fertility begins in puberty (Miller, 1994a; Johnson & Everett, 1995). In the female, the post pubertal changes resulting in adult reproductive system manifest in cyclic patterns of ovarian development consisting of follicular maturation, ovulation and formation and regression of the corpus luteum, collectively known as the ovarian cycle. These processes ensure the release of matured, or developed female germ cells (oocytes) capable of fertilisation. Concurrent secretion of steroids enhances the ovarian development and prepares the female reproductive tract for pregnancy. The release of matured oocytes and secretion of steroids are the two main roles of an adult ovary (Fertum et al., 1994; Johnson & Everett, 1995). The structure of the ovary (Fig. 1.1) is developed to fulfil these roles, which are principally controlled by endocrine action of the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH), acting from the pituitary (Zelinski, 2001). It is the control of ovarian function, particularly the regulation of 5 α -reductase metabolism that is investigated in this thesis.

The ovarian steroid responsible for steroid production are the thecal and granulosa cells within the follicles (Hart-Langton & Armstrong, 1988). It is essential

Chapter 1. LITERATURE REVIEW

1.1. INTRODUCTION

Androgens are arguably the most important ovarian steroids because they are the precursor substrates from which oestrogens are synthesised (Hillier, 1981; Adashi, 1994; Hillier *et al.*, 1994; Shoham & Schachter, 1996). In addition to this main role, androgens also have local paracrine functions where they modulate follicle development via regulatory action on key steroidogenic enzymes (Hillier & De Zwart, 1981; Harlow *et al.*, 1986). Ovarian androgens are, therefore, cardinal in ovarian function on which female fertility is critically dependent. This thesis focuses on the intraovarian role of androgens, specifically 5 α -reduced androgens that are produced as a result of the catalytic action of 5 α -reductase (5 α -R), in normal and abnormal ovarian function.

Ovarian androgen secretion begins *in utero* (Levina *et al.*, 1976; Grinsted, 1982), but the production of female sex steroids, which regulate fertility, begins at puberty (Hillier, 1994a; Johnson & Everitt, 1995). In the female, the post pubertal changes heralding an adult reproductive system manifest in cyclic patterns of ovarian developments consisting of follicular maturation, ovulation, and formation and regression of the corpus luteum, collectively known as the ovarian cycle. These processes ensure the release, at ovulation, of developed female germ cells (oocytes) capable of fertilisation. Concomitant secretion of steroids enhances the ovarian developments and prepares the female reproductive tract for pregnancy. The release of fertilisable oocytes and secretion of steroids are the two main roles of an adult ovary (Tsafri *et al.*, 1994; Johnson & Everitt, 1995). The structure of the ovary (Fig. 1.1) is developed to fulfil these roles, which are principally controlled by endocrine action of the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH), arising from the pituitary (Zelevnik, 2001). It is this control of ovarian function, particularly, the regulation of 5 α -reductive metabolism that is investigated in the studies described in this thesis.

The ovarian tissues responsible for steroid production are the thecal and granulosa cells within the follicles (Gore-Langton & Armstrong, 1988). It is essential

that the levels of bioactive androgens required for normal ovarian function are kept within physiological ranges because overproduction of these steroids is strongly linked with states of infertility arising from dysfunctional follicle development and multiple disorders of health associated with polycystic ovarian disease. Because of this association, part of the studies described in this thesis analysed the levels of androgens and oestrogens in follicular fluids from normal and polycystic ovaries. The purpose of this chapter is to review the relevant literature used to set the scene for the laboratory and clinical studies that follow.

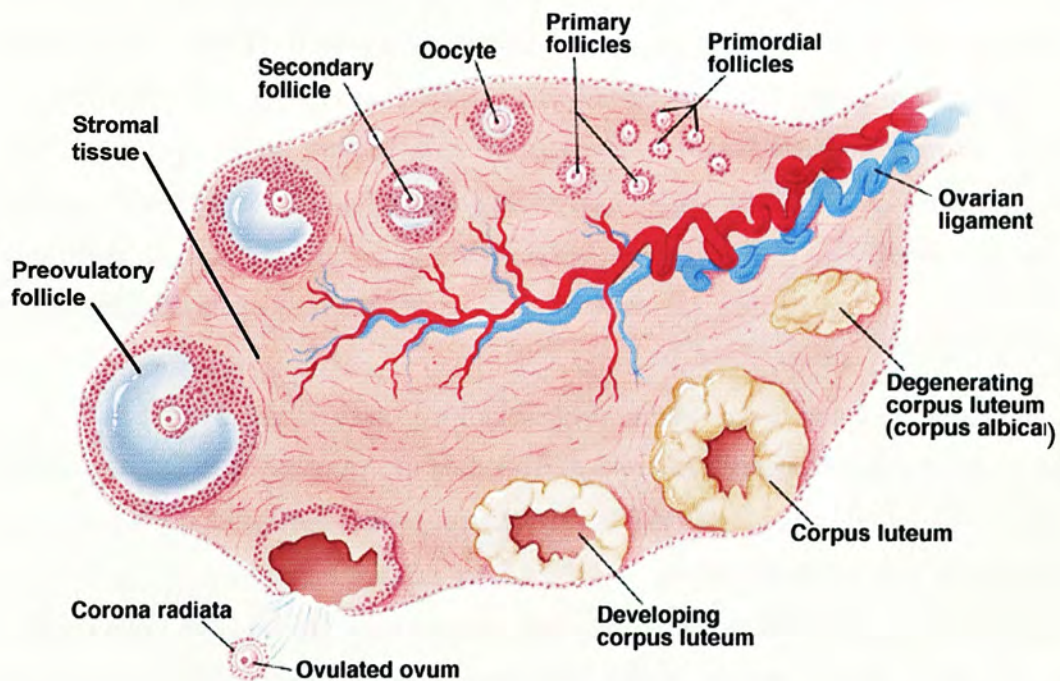


FIGURE 1.1. Structure of adult ovary. The follicle is the gametogenic and steroidogenic engine of the adult ovary. The ovary contains stromal tissue in which the primordial follicles containing the female eggs (oocytes) and glandular tissue (interstitial tissue), are embedded. The primordial follicles develop *in utero* and begin to develop into primary and secondary follicles, which only develop further after puberty into larger antral and then preovulatory follicles containing mature oocytes. At ovulation, a developed egg (ovum) is released from the preovulatory follicle (in monovulatory species) with a residual layer of granulosa cells surrounding it (corona radiata). The collapsed follicle develops into a yellowish corpus luteum, which is the steroid-producing tissue of the ensuing luteal stage of the ovarian cycle. Regression of the corpus luteum leaves a whitish scar tissue, the corpus albicans, which is absorbed into the stromal tissue of the ovary over a period that varies from weeks to months, depending on the species. *Adapted from Byer/Shainberg/Galliano. Dimensions of Human Sexuality, 5e.1999. The McGraw-Hill Companies, Inc.*

1.2. OVARIAN FUNCTION

1.2.1. Ovarian Follicle Development

Fertility in the adult female is dependent on oocytes that develop in the ovaries during fetal development *in utero* (Wassarman, 1988). Development of the oocytes halts *in utero* (human, cow, sheep, goat, mouse) or ends just after birth (rat, pig, cat, rabbit, hamster) (Johnson & Everitt, 1995). At this stage, the ovaries contain mainly primordial follicles bearing oocytes in a state of meiotic arrest.

The somatic (granulosa and thecal) cells surrounding these oocytes subsequently undergo developmental changes that transform primordial follicles into primary, secondary (preantral) stages, and then antral follicles, which require post-pubertal levels of gonadotrophins for further development (Greenwald & Terranova, 1988; Johnson & Everitt, 1995; McGee & Hsueh, 2000). The duration of these stages of development differs among species, but the preantral stages are invariably longer than the antral stages. The sequence through which a primordial follicle develops into a mature, fully developed follicle that releases an oocyte ready for fertilisation, constitute the process called folliculogenesis (Fig. 1.2).

In man, the entire process of folliculogenesis takes more than 6 months whereas in rat, it is less than 2 months. In both species, it occurs throughout life until the stock of oocytes is exhausted (Gougeon, 1982; McGee & Hsueh, 2000). The initial recruitment of primordial follicles into a pool of growing follicles is a continuous process that probably begins just after the formation of the follicles, long before puberty (McGee & Hsueh, 2000). As some primordial follicles initiate growth, others remain quiescent for months or years. While attempts are being made to elucidate why primordial follicle development begins (Vendola *et al.*, 1999b; Fortune *et al.*, 2000), the trigger of this initial development is not known. However, it is now known that the process is gonadotrophin-independent (Hillier, 1994a; McNatty *et al.*, 1999), and possibly due to intraovarian and/or other unknown factors. Recruited follicles that reach the secondary or early antral phases before puberty become atretic. Therefore, folliculogenesis only becomes a fully gonadotrophin-controlled process at puberty (Baird, 1991).

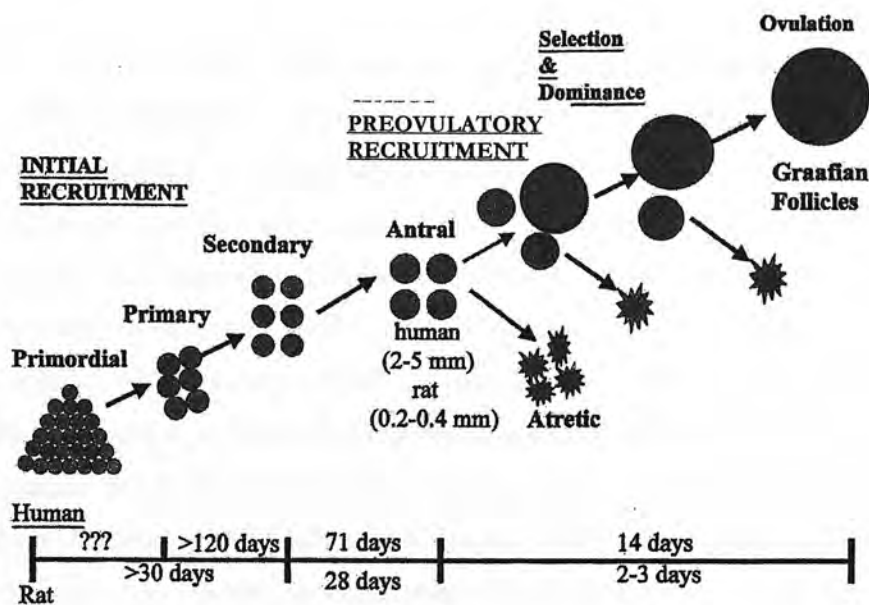


FIGURE 1.2. Stages and duration of folliculogenesis in human and rat ovaries. Primordial follicles undergo initial recruitment to enter the growing pool of follicles which develop into primary, secondary and then antral follicles. Further development of antral follicle occurs after onset of puberty when gonadotrophins begin cyclic stimulation of small antral follicles that grow into larger antral follicles from which one or a few follicles (dependent on the species) are “selected” during dominance for further development into Graafian (preovulatory) follicles from which mature ova are released at ovulation. The whole process of folliculogenesis takes longer than 6 and 2 months in man and rat, respectively. (Adapted from McGee and Hsueh, 2000)

1.2.1.1. Preantral follicle development

During preantral growth, as the follicle increases in size, major growth occurs in the oocyte, which reaches a final diameter of 60-120 μm (Johnson & Everitt, 1995). At these stages, oocytes remain "meiotically arrested", but are metabolically active in synthesis of ribonucleic acids (RNA) and proteins in preparation for subsequent post-ovulatory developmental changes (Greenwald & Terranova, 1988; Johnson & Everitt, 1995). As the follicle develops, the oocyte forms a translucent acellular, glycoproteinaceous layer around itself called the zona pellucida. The zona pellucida separates and protects the oocyte from the layers of granulosa cells that develop around it. Cytoplasmic processes penetrating through the zona pellucida from surrounding granulosa cells allow communication between the oocyte and the proliferating granulosa cells. As the oocyte grows in size, the granulosa cells proliferate and become multi-

layered. The preantral follicle also increases further in size due to the condensation of ovarian stromal cells on the outside of the follicle. This enveloping matrix of spindle-shaped cells gives rise to the thecal layer of the follicle. By undergoing further development and proliferation, the thecal and granulosa cells grow as two distinct follicular layers separated by a basement membrane. These two cell types are the steroidogenic engines of the ovary (Fig. 1.3). The thecal layer develops further, becoming richly interlaced with blood vessels while the granulosa layer remains avascular. Further development of the granulosa cell layers results in the accumulation of a thick viscous serum exudate known as follicular fluid. The coalescence of this follicular fluid, comprised mainly of mucopolysaccharides secreted from granulosa cells, creates an antrum that marks the beginning of the antral phase of follicular development (Greenwald & Terranova, 1988; Johnson & Everitt, 1995). It is follicles at this stage that are steered into further preovulatory developments by adult levels of gonadotrophins.

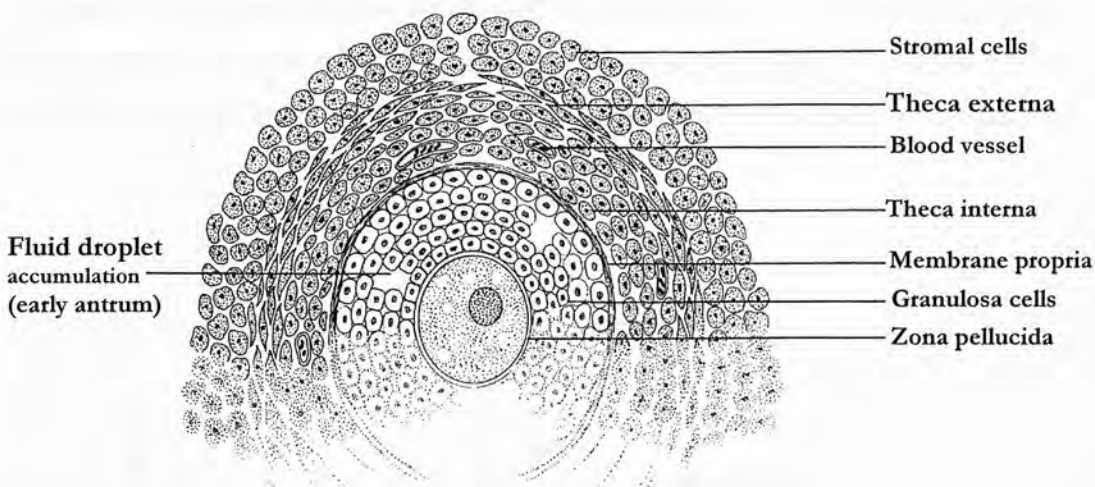


FIGURE 1.3. Early antral follicle. The granulosa and thecal cells are proliferated with thecal cells now comprising of two layers, an outer fibrous externa and an inner theca interna, rich in blood vessels. Coalescing follicular fluid drops are evident within the granulosa cells. (*Adapted from Johnson & Everitt, 1995*)

Although the preantral stages of follicular development are gonadotrophin-independent, antrum formation is evidently initiated by FSH *in vitro* (Gutierrez *et al.*, 2000) and *in vivo* (Hillier *et al.*, 1980c; Gougeon, 1982). This endocrine action is

supplemented by ovarian paracrine factors, which in turn are regulated by FSH (Hillier, 1999;2001). Circulating levels of FSH required to begin antrum formation and recruit preantral follicles into the small antral stages are adequate throughout childhood.

The onset of puberty, characterised by increased secretion of gonadotrophins by the anterior pituitary gland, occurs when follicle development proceeds to the cyclic preovulatory type of recruitment, which culminates in the release, at ovulation, of meiotically developed mature oocytes capable of fertilisation.

1.2.1.2. Cyclic follicle development

The cyclic release of fully developed oocytes capable of fertilisation and the follicular production of sex steroids that regulate female reproduction are the hallmarks of the normal functioning adult ovary (Tsafriri *et al.*, 1994; Johnson & Everitt, 1995). The length of the ovarian cycle varies between species, taking about 28 and 4 - 5 days in man and rodents, respectively, but the gonadotrophin-induced ovarian events are similar as illustrated in Fig. 1.2. In man, the end of the ovarian cycle, as marked by the regression of the corpus luteum, is followed by disintegration of the developed uterine lining which is lost from the body during menstruation, and hence the ovarian cycle is called the menstrual cycle in women. In species where menstruation does not occur, it is

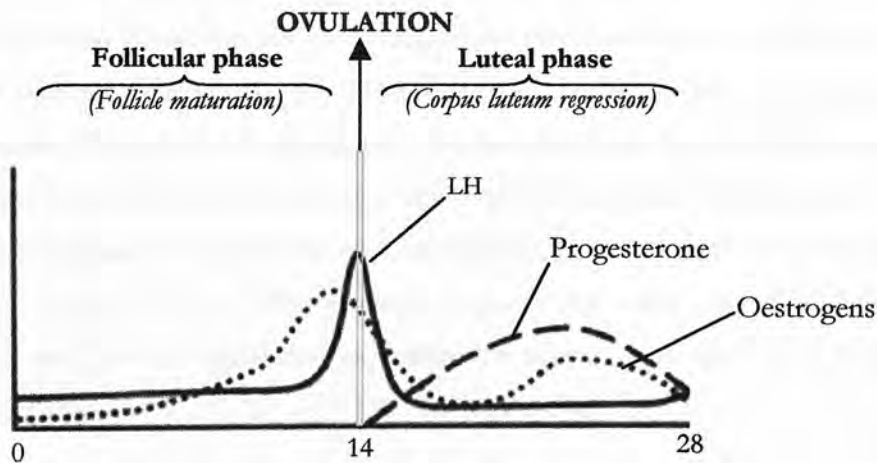


FIGURE 1.4. Summary of follicular activity in the menstrual cycle. On average, the menstrual cycle takes 28 days. The pivotal position of ovulation separates the cycle into a first half (follicular phase) which is essentially an oestrogen-secreting phase and a second half (luteal phase) which is a progesterone-secreting phase. Functionally, the two phases are responsible for follicle maturation, and regression of the corpus luteum, respectively. Luteinising hormone (LH) is the endocrine trigger for ovulation. The relative blood hormones levels are as indicated. (*Adapted from Johnson & Everitt, 1988*)

referred to as the oestrous cycle. The follicular phase of the ovarian cycle preceding ovulation takes approximately 14 days and 2 - 3 days in man and rat, respectively (Johnson & Everitt, 1995; McGee & Hsueh, 2000). A scheme showing the major endocrine events associated with the human menstrual cycle is shown in Fig. 1.4. The transformation of the immature (preantral/early antral) follicles into preovulatory follicles is also associated with dramatic changes in the synthesis and metabolism of androgens, which are the main focus of this thesis.

I. Preovulatory recruitment of antral follicles

Small increases in the levels of FSH mark the beginning of the ovarian cycle (Baird, 1991; Hillier, 1991a), resulting in the recruitment of small antral follicles into preovulatory stages of development. This advanced antral stage is predominantly a phase of extensive growth which is mainly due to great increases in the size of the follicular antrum (Fig. 1.5). Proliferation of granulosa and thecal cells, due to gonadotrophic stimulation, also contributes to the preovulatory increase in follicle size (Greenwald & Terranova, 1988). Generally, FSH is the gonadotrophin responsible for initiation of development of the preantral follicles in most species (Hillier, 1991b; Zelinski-Wooten et al., 1995; Gutierrez *et al.*, 2000), but there are indications that LH also probably has a role during this period to prime thecal cells in preantral follicles for subsequent development-related responses (Wu *et al.*, 2000).

Thecal and granulosa cells show differential responsiveness to FSH and LH as the follicle matures. Throughout the follicle life-cycle, thecal cells possess receptors for LH, and hence respond to LH stimulation. On the other hand, granulosa cells respond to FSH because of their preferential expression of FSH receptors (Hillier *et al.*, 1994). However, during advanced preovulatory development, granulosa cells also develop LH receptors. Thus, whereas FSH generally induces the early stages of follicular recruitment and growth-related changes within the ovary, LH is required to complete them (Spears *et al.*, 1998).

Within the cohort of growing preovulatory follicles, FSH increasingly induces further granulosa cell proliferation and antrum expansion (Hillier, 1994a; b). This mitogenic action is enhanced by locally produced oestrogens, particularly oestradiol, the increased production of which becomes the hallmark of the follicle destined to ovulate.

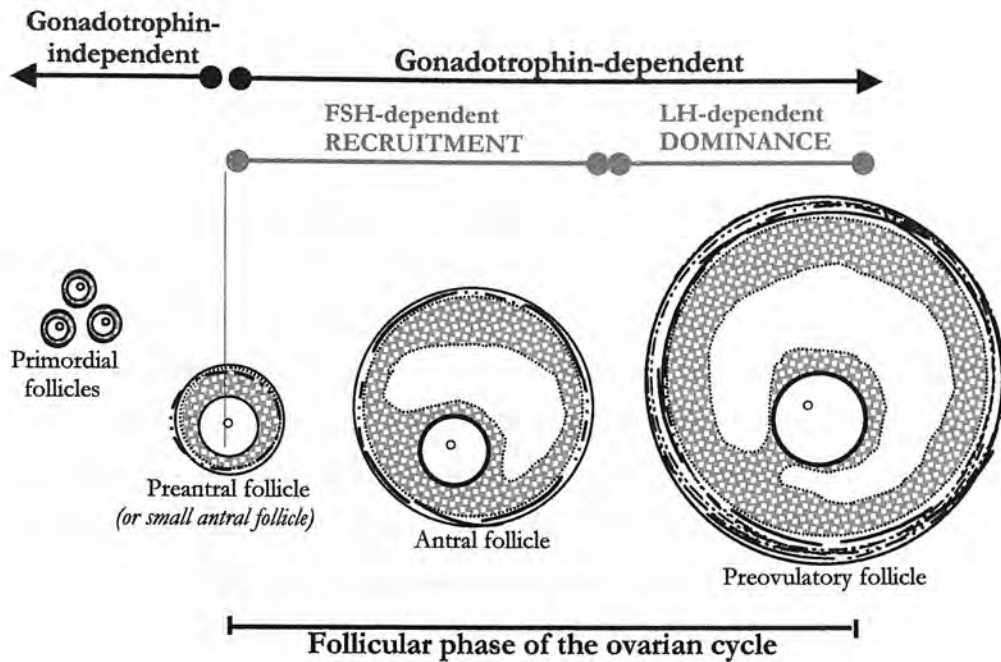


FIGURE 1.5. Follicle development during follicular phase of the ovarian cycle. During the ovarian cycle, transformation of preantral follicles into preovulatory follicles, which are released at ovulation occurs through a series of developmental stages termed the follicular phase of the ovarian cycle. Progression through the follicular phase is principally regulated by gonadotrophic signals, follicle stimulating hormone (FSH) and luteinising hormone (LH), which recruit follicles for development and complete follicular maturation through selection of usually one dominant follicle in monovulatory species, for example, humans and sheep. (*Adapted from Hillier, 1994a*)

Due to the specific response to gonadotrophins exhibited by granulosa and thecal cells at this stage, thecal cells respond to LH and synthesise androgens, which are converted to oestrogens within the granulosa cells (Hillier, 1994a,b). By creating this local positive-feedback loop, the thecal-granulosa co-operative action amplifies oestrogen production, which enhances the FSH-induced stimulation of follicle proliferation. As a result, the developing follicles are stimulated by both endocrine and paracrine signals, which enhance follicle development.

The number of follicles selected for ovulation varies among species and is species-specific (Baird, 1991). In multiovulatory species like rats and pigs, development of several follicles occurs synchronously and as a result, multiple ovulation occurs. In monovulatory species such as man, single follicles are usually selected for further development while the others become atretic. Only the dominant follicle(s) selected from among the recruited follicles grows further to ovulatory or near-ovulatory size.

Selection and dominance are accompanied by further increases in the ability of thecal cells to produce androgens and granulosa cells to aromatise them to oestradiol. Hence, dominant follicles secrete steroids that ensure their continued development.

Locally, oestrogens are important for another crucial development in the follicles. Together with FSH, oestrogens induce the expression of LH-receptors on the outer layers of granulosa cells, which hitherto lacked them (Richards *et al.*, 1987). This is vital for the progression of the dominant follicle(s) into the final preovulatory stages of development prior to ovulation (Figs 1.2 and 1.4). In the face of decreasing FSH stimulation, the dominant follicles continue to develop because they have acquired the capacity to respond to LH (Hsueh *et al.*, 1984; Sullivan *et al.*, 1999). The switch from dependence on FSH to LH-responsiveness indicates that the dominant follicle(s) is ready to enter the final preovulatory phase in preparation for ovulation.

II. Final development of preovulatory follicles

The preovulatory stage of follicle development is characterised by dependence on LH and growth of the dominant follicle. Progression of the dominant follicle to ovulation is dependent on a brief surge of very high levels of LH (see Fig. 1.4), which provides the impetus that pushes the dominant follicle through the final stages of growth before the expulsion of the oocyte from the follicle at ovulation. The follicle structure breaks down and the pattern of steroid secretion also changes. The LH surge is crucial because absence of the transient LH peak results in the death of the expanded follicle which manifests as a failure to release a developed ovum, a condition known as anovulation.

The surge coincides with the expression of LH receptors on the outer granulosa cells of the dominant follicle(s) such that, aromatase, the key enzyme in ovarian endocrinology, becomes functionally coupled to LH at this stage (Adashi, 1994; Zeleznik & Hillier, 1984; Richards, 1994). As a result, LH stimulates both androgen synthesis and aromatisation in dominant follicles, thereby directly stimulating oestrogen production. The dominant follicle produces more androgens than the non-dominant follicles within its cohort due to its need for higher amounts of substrate for conversion into oestrogens. The exposure to high LH levels is only beneficial to follicle growth at

this stage of folliculogenesis because premature elevation of LH induces atresia or impairs oocyte development in immature follicles (Chappel & Howles, 1991; Filicori, 1999).

The LH surge is also responsible for the endocrine changes that occur in the follicle and continue after ovulation. At the time of the transient LH rise, the granulosa layer has also undergone changes in its steroidogenic activity. Because they express LH receptors at this stage (Hsueh *et al.*, 1984; Hickey *et al.*, 1990), granulosa cells, together with the thecal cells, respond to LH during the LH surge and switch to synthesising progesterone instead of aromatising androgens to oestrogens. The LH surge, which initially stimulates thecal cell activity, subsequently stops the endocrine action of the thecal cells. Therefore at this stage, LH is bifunctional in that it stimulates steroidogenesis in the granulosa cells of the ruptured follicle, but inhibits further cell proliferation (Hugues & Cedrin-Durnerin, 2000).

The structural changes that occur concurrently with the LH surge enhance the steroidogenic function within the ovary. As the basement membrane breaks down and blood vessels invade and infiltrate the disrupted granulosa cell layers (a process called angiogenesis), acetate and cholesterol are carried into the granulosa-stromal-thecal matrix which gives rise to the corpus luteum, the predominant structure in post-ovulation ovaries (Johnson & Everitt, 1995). At this stage, the expression of FSH receptors on granulosa cells is decreased. However, due to the expression of LH receptors on luteinised granulosa cells of the corpus luteum, this stage is predominantly a phase of LH-induced progesterone synthesis (Adashi, 1994; Tsafiriri *et al.*, 1994). In some species like the rat and mouse, luteinised granulosa cells also acquire prolactin receptors because prolactin is essential for the maintenance of the luteal phase in these animals (Johnson & Everitt, 1995).

Subsequent developmental changes in ovarian follicles depend on whether or not fertilisation takes place. In man, if fertilisation and implantation occur, the ovary continues producing progesterone in response to stimulation by trophoblast-derived choriogonadotrophic hormone, which is necessary for the maintenance of pregnancy. In the absence of fertilisation, the luteal phase terminates, heralding a new ovarian cycle (McGee & Hsueh, 2000). Therefore, oocyte development and steroidogenesis are

coupled functions of the adult ovary. In some species like the rat and mouse, the length of the cycle depends on whether or not the animal mates. In the absence of mating at the time of ovulation, the luteal phase takes 2-3 days. It can be longer, 11-12 days (pseudopregnancy) if the female has an infertile mating.

It is also worthwhile noting that besides the regulatory interplay between the steroidogenic cells, paracrine communication between the oocyte and granulosa cells is crucial during folliculogenesis for the formation of the antrum, granulosa cell steroidogenesis, and for the maturation of the oocyte (Reynaud *et al.*, 2000). Other endocrine factors also affect folliculogenesis. Growth hormone, produced in the pituitary, acts directly on the ovary where it stimulates steroidogenesis in granulosa cells *in vivo* or *in vitro*, thereby acting as a co-gonadotrophin (Franks, 1998). Kawagoe & Hiroi (1990) showed that prolactin, another pituitary hormone modulates steroidogenesis and follicle development of small follicles.

1.2.2. Ovarian Steroidogenesis

As described above, ovarian follicles respond to gonadotrophic stimuli and concurrently produce increasing amounts of steroids while undergoing follicle development. The antral stages of follicular development are by far the most important source of ovarian steroids during the female cycle (Gore-Langton & Armstrong, 1988). Steroids are lipids with structures based on the perhydrocyclopentane-phenanthrene fused ring system (Lieberman *et al.*, 1984; Strauss & Miller, 1991; Granner, 1996). In mammals, most of the commonly occurring steroids are molecules containing between 18 and 27 carbon atoms, which are related to or derived from cholesterol. The mammalian ovary produces all three major classes of steroids; oestrogens, progestagens and androgens, which are products of the same biosynthetic pathway (Fig. 1.6; Hsueh *et al.*, 1984; Gore-Langton & Armstrong, 1988; Strauss & Miller, 1991). It is worth noting that the preovulatory follicle is not an important source of circulating progestagens because ovarian steroidogenesis at this stage is geared towards synthesising oestrogens from androgenic precursors until ovulation occurs.

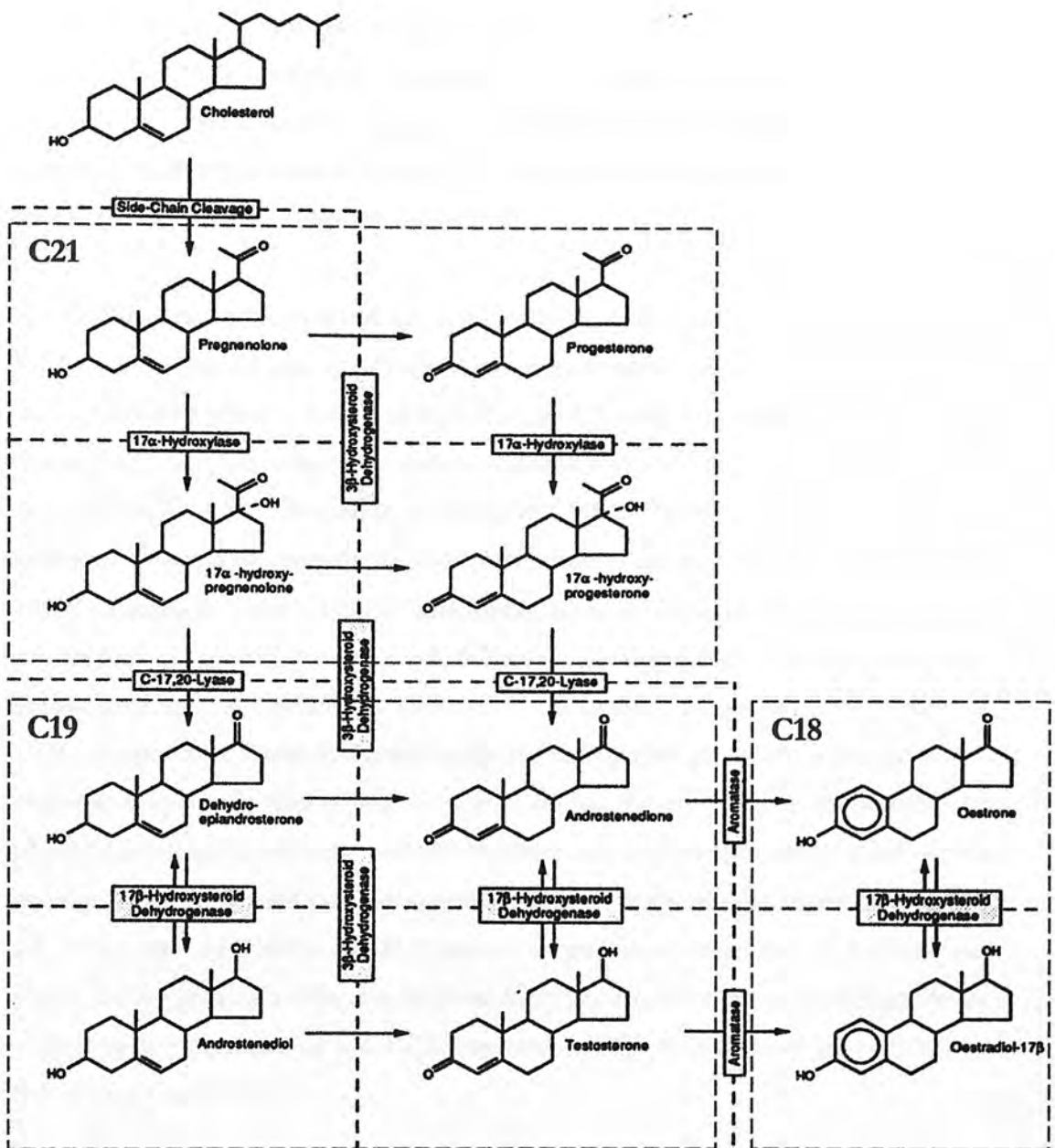


FIGURE 1.6. Pathways of steroid synthesis. The major classes of ovarian steroids are synthesised in the same pathway which originates from cholesterol. Progestogens (C21 steroids) are converted into androgens (C19 steroids) which are aromatised into oestrogens (C18 steroids). (*Adapted from Strauss & Hillier, 1991a*)

1.2.2.1. Cellular sources of ovarian steroids

I. Thecal and interstitial cells

Thecal cells express enzymes that perform *de novo* synthesis of steroids (oestrogens and androgens) from acetate and cholesterol, but the predominant expression of the 17 α -hydroxylase/C17,20 lyase enzyme complex, the rate-limiting enzyme in the biosynthesis of androgens, encoded by the CYP17 gene (CYP17),

essentially specifies thecal cells as androgen producers (Strauss & Miller, 1991; Adashi, 1994). The main androgens produced in the ovary are androstenedione and testosterone. The expression of high density lipoprotein (HDL) receptors on thecal and interstitial cells, while absent on granulosa cells, enables these cells to use circulating cholesterol for steroid synthesis (Li *et al.*, 1998).

It is now well known that LH is the endocrine signal that thecal cells respond to for the production of androgens from the precursor substrates, acetate and cholesterol during the antral phase of follicle growth (Hugues & Cedrin-Durnerin, 2000). Signalling through LH receptors which are present constitutively on thecal cells, and mediated through cyclic AMP (Bogovich & Richards, 1984; Hillier, 1985), LH stimulates androgen secretion by upregulating 17 α -hydroxylase/17,20 lyase (Bogovich & Richards, 1982; Johnson & Crane, 1995). The ability to metabolise cholesterol for steroid production is related to stage of follicular development. Equine chorionic gonadotrophin (eCG), which has FSH and LH bioactivity, increases the expression of HDL receptors in thecal/interstitial cells indicating that gonadotrophins stimulate cholesterol uptake by thecal/interstitial cells during the early stages of the follicular phase. Increased cholesterol uptake provides the steroid precursor from which androgens are synthesised via progestogens in thecal/interstitial cells, as indicated in Fig. 1.6. Only after expression of LH receptors on granulosa cells, later in the follicular phase, do the granulosa cells also respond to LH and begin the cholesterol uptake as shown by the presence of the HDL receptor mRNA in luteinised granulosa cells (Johnson & Crane, 1995).

II. Granulosa Cells

Mature granulosa cells express several steroidogenic microsomal enzymes. The major one is aromatase cytochrome P450 (P450_{arom}), which is a product of the CYP19 gene (Richards, 1994; Simpson *et al.*, 1994) that synthesises oestrogens (see Fig. 1.6). Granulosa cells also express other enzymes that synthesise progestins, pregnenolone and progesterone *de novo* from acetate and precursor steroids (Hsueh *et al.*, 1984; Richards, 1994). However, granulosa cells of all species studied, with the apparent exception of the bovine (Lacroix *et al.*, 1974) do not have significant 17 α -hydroxylase/17,20 lyase activity and as a result, synthesise little or no androgens from either pregnenolone or progesterone (Gore-Langton & Armstrong, 1988).

The main oestrogens produced in the ovary are oestradiol and oestrone, which are produced by aromatisation of the androgens secreted by the thecal cells. Granulosa cells secrete oestrogens in response to FSH, which acts through its receptors that are constitutively present on the cells (Hillier, 1985; Adashi, 1994; Richards, 1994; McGee & Hsueh, 2000). In some species such as pigs, chickens and tree shrews, oestrogens are also synthesised by thecal cells (Rosenfeld *et al.*, 2001). However, in most species, man included, it is the transfer of androgens from theca and granulosa cells that is responsible for the oestrogen in the follicular fluid and most of the oestrogen found circulating in blood.

Another major steroid enzyme expressed by granulosa cells is 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1; (Makris & Ryan, 1980; Nimrod *et al.*, 1980; Moon & Duleba, 1982). Belonging to a class of enzymes with reversible hydroxysteroid/dehydrogenase and ketosteroid/reductase (HSD/KSR) activities, 17 β -HSD1 inter-converts 17-keto and 17-hydroxy steroids (see Fig. 1.6) (Penning, 1997; Peltoketo *et al.*, 1999). Both oestrogens and androgens have the highest affinity for their receptors in the 17 β -hydroxy form. Therefore, testosterone and oestradiol are more potent than androstenedione and oestrone, respectively. Hence, 17 β -HSD1 regulates the biological activity of the sex hormones. Although androstenedione is the major ovarian 17-keto androgen in most species, the ovarian 17 β -HSD isozyme, 17 β -HSD1 favours the production of oestradiol as the main ovarian oestrogen, thereby, making ovarian 17 β -HSD1 essentially an enzyme for oestradiol production.

Thus, due to the enzymes expressed in the thecal and granulosa cells, various steroids are secreted in the follicular fluid, but only the steroid class of main interest to this thesis, androgens, is reviewed extensively here.

1.2.2.2. Ovarian androgens

Androgens are C₁₉ compounds synthesised by the steroid biosynthetic pathway shown in Figs. 1.6 and 1.7 (Gore-Langton & Armstrong, 1988; Strauss & Miller, 1991). Ovarian androgens are synthesised from the C₂₁ steroids by the afore-mentioned, rate-limiting enzymes, 17 α -hydroxylase/C17-20 lyase that are present in a single steroidogenic cytochrome P450c17 (P450c17) encoded by the CYP17 gene (Fevold *et*

al., 1989; Draincourt *et al.*, 1993). In ovarian follicles, the CYP17 gene is expressed only in thecal/interstitial cells (Smyth *et al.*, 1993), which explains why ovarian androgens are exclusively synthesised by these cell types.

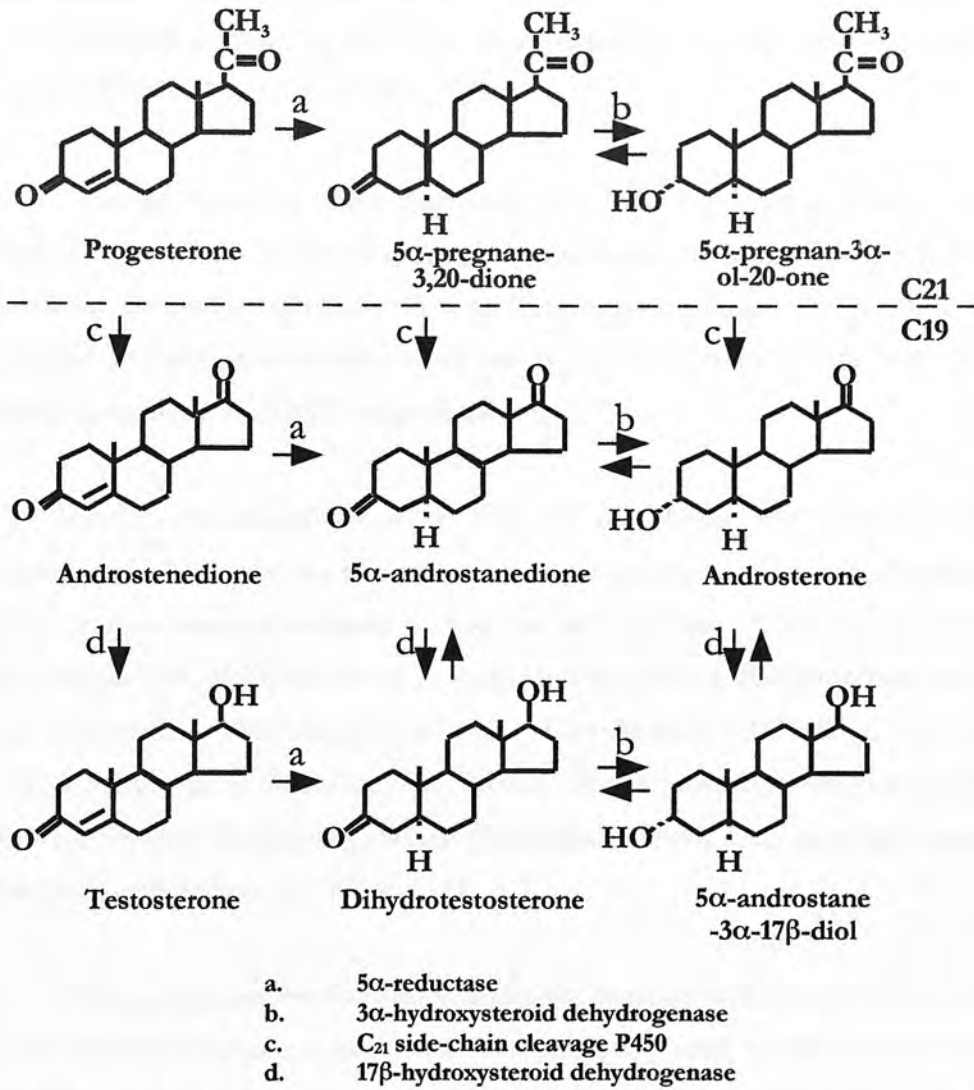


FIGURE 17. Biosynthetic pathway of androgens. Dihydrotestosterone and androstenedione are also metabolised to 5 α -androstane-3 β ,17 β -diol and epiandrosterone, respectively by 3 β -hydroxysteroid dehydrogenase. (Adapted from Gore-Langton & Armstrong, 1998).

The main C₂₁ steroids available for the 17 α -hydroxylase/C17-20 lyase reaction, as shown in Fig. 1.6, are progesterone and pregnenolone. They are metabolised to androstenedione, and dehydroepiandrosterone (DHEA), respectively, which are consequently the major C₁₉ steroids produced by the ovary. DHEA is a substrate for

the enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSD) which catalyses the conversion of Δ^5 -3 β -hydroxy steroids into the corresponding Δ^4 -3 β -oxo steroids (Fig. 1.6). As a result, androstenedione (androst-4-ene-3,17-dione), the major androgen secreted by the human ovary (McNatty *et al.*, 1979a) can be produced by both the Δ^4 (progesterone \rightarrow androstenedione) and Δ^5 (pregnenolone \rightarrow DHEA \rightarrow androstenedione) routes of biosynthesis (Miller, 1988). In the ovary, androstenedione is converted to testosterone by 17 β -HSD1 (Andersson *et al.*, 1995).

Other quantitatively minor androgens with indistinct biological roles are also produced in the ovary. These are either precursor steroids in the androstenedione and testosterone steroidogenic pathways, i.e., DHEA and androstenediol, respectively (Fig. 1.6), or the 5 α -reduced metabolites of the two steroids i.e., 5 α -androstenedione (5 α -A) and dihydrotestosterone (DHT), respectively (Fig. 1.7).

Besides the steroid-secretory roles of the thecal and granulosa cells, "communication" between the two cell types is necessary for intraovarian regulation of normal ovarian function mediated by both steroidal (Hillier, 1987; Ainsworth *et al.*, 1990; Conway *et al.*, 1990; Fitzpatrick & Richards, 1991; Hillier, 2001) and non-steroidal (Cara & Rosenfield, 1988; Adashi *et al.*, 1991; Hillier & Miro, 1993; Wood *et al.*, 1994; Mason & Franks, 1997; Slee *et al.*, 2001) factors. The intraovarian paracrine regulation of ovarian function modulates gonadotrophin action, thereby increasing the sensitivity of the developing follicles to FSH and LH.

Although this review focuses on androgen function within the ovary, steroids are also important endocrine hormones. Steroids are carried by blood to peripheral tissues, for example, breast, prostate, and uterine where they are metabolised by local enzymes into bioactive steroid hormones (Labrie, 1991; 1995). By creating tissue/cellular steroid-producing environments that are distinct from the main steroid-producing organs, namely, the gonads and adrenals, the steroidogenic and steroid metabolising enzymes generate intracrine hormone action where the locally produced steroids are physiologically active. This intracrine production of steroid hormone accounts for 75% of bioactive steroid in premenopausal women, approximately 100% in postmenopausal women, and approximately 50% of androgens in men (Labrie, 1991;

Labrie *et al.*, 2000b). Examples of enzymes involved in intracrinology are 5 α -R in skin which converts testosterone to DHT that stimulates hair growth locally (Azziz *et al.*, 2000; Labrie *et al.*, 2000a), and 17 β -HSDs which catalyse the last and key step in the formation of all estrogens and androgens (Labrie *et al.*, 2000b). Intracrinology is key to understanding steroid biosynthesis and action in peripheral tissues. The remainder of this review is limited to the paracrine role of intraovarian androgens.

1.3. PARACRINE ACTION OF ANDROGENS

Paracrine action takes place where cellular products are secreted and affect neighbouring cells, the secretory cells themselves not being affected due to lack of suitable receptors (Murray, 1996). Because the thecal and granulosa cells perform the steroidogenic function of the ovary, the paracrine/autocrine regulations are “liaisons” between the two cell types, as illustrated by Smyth *et al.*, (1995). Paracrine action of androgens on granulosa cells is exerted through androgen receptors that are located in the nuclei of granulosa cells (Schreiber & Ross, 1976; Hillier & Tetsuka, 1997; Szoltys & Slomczynska, 2000).

1.3.1. Paracrine Action of Androgens on Ovarian Follicle Development

In immature rats, LH-induced androgen action enhanced follicular atresia by antagonising oestrogen-enhanced follicle development (Louvet *et al.*, 1975; Ross, 1985). Therefore, in immature rats, the paracrine action of androgens suppresses the FSH-induced effects of oestrogen action. In addition, androgens decreased the number of granulosa cells per follicle *in vivo*, of eCG-stimulated rats (Conway *et al.*, 1990). The involvement of the androgen receptor in these androgenic effects was illustrated in granulosa cells where DHT down-regulated the expression of androgen receptor mRNA expression in immature granulosa cells via a mechanism that was reversible by FSH (Tetsuka & Hillier, 1996). Therefore, in immature rats, the androgen-induced inhibitory effects on follicle growth are mediated through the androgen receptor, which is also regulated paracrinally by androgens as shown by their down-regulation of androgen receptor mRNA and protein (Tetsuka *et al.*, 1995; Hillier *et al.*, 1997).

It is important to point out that intraovarian paracrine androgen action is not limited to granulosa and thecal cells. Androgens also affect ovarian stromal tissue

because they induce thecal hyperplasia and capsular thickness in immature follicles, in the short term (Vendola *et al.*, 1998). This stimulatory role is supported by clinical evidence where androgen-producing tumours, congenital adrenal hyperplasia (an androgen-secreting condition) and exogenous androgen treatment are associated with the development of hypertrophic polycystic ovary (PCO) phenotypes. The mechanism(s) whereby androgens stimulate ovarian growth are still unclear. Some of the intermediary factors thought to be involved are members of the IGF family, IGF-1 in murines (Adashi, 1998), and IGF-II in man (Bondy *et al.*, 1993) and pigs (Zhou *et al.*, 1996). By stimulating the production of these IGFs (Vendola *et al.*, 1999a; Vendola *et al.*, 1999b), which are known to enhance oestrogen production (Wood *et al.*, 1994) and follicle development (Yong *et al.*, 1992), androgens probably modulate follicle growth indirectly.

Androgens, therefore, decrease follicle growth and development by inhibiting FSH-mediated action on granulosa cells, but stimulate hypertrophy of stromal tissue.

1.3.2. Paracrine Action of Androgens on Ovarian Steroidogenesis

Due to the critical role of steroids in reproduction, it is not surprising that there are multiple regulatory systems available to modulate their production and release. Although aromatisable androgens induce atresia in the absence of FSH, they augment gonadotrophin-stimulated steroidogenesis in its presence (Hsueh *et al.*, 1984; Conway & Mills, 1991). For example, testosterone increased oestradiol synthesis *in vitro* by a synergistic interaction with FSH (Hillier & De Zwart, 1981). Therefore, androgens enhance FSH-stimulated aromatase activity not only by acting as substrates for oestrogen production, but also by exerting direct paracrine effects which up-regulate the activity of this steroidogenic enzyme. It is thought that the mechanism of androgenic paracrine action is mediated via cyclic AMP, the formation of which is stimulated by androgens in the presence of FSH (Knecht *et al.*, 1984; Hillier & Tetsuka, 1997). Androgens, therefore, enhance granulosa cell production of cAMP via which FSH actions are mediated.

Intracellular cAMP is the major second messenger that mediates FSH action in granulosa cells (Hillier & Tetsuka, 1997). By inducing post-receptor signalling within the cells, cAMP provides the link between the extracellular FSH signal and the

intracellular responses. Although androgens do not stimulate cAMP formation in the absence of FSH, in its presence, androgens are stimulatory (Hillier & Tetsuka, 1997).

Aromatisable androgens are more potent paracrine steroids than non-aromatisable androgens (Daniel & Armstrong, 1983). Both types of androgens also exhibit different paracrine action on aromatase activity. While the major aromatisable androgens, testosterone and androstenedione enhanced oestradiol production, the non-aromatisable androgen, DHT suppressed oestradiol production by inhibiting FSH-induced expression of LH receptors on granulosa cells (Farookhi, 1980), thereby illustrating that DHT inhibits the production of oestrogens from preovulatory follicles.

On the other hand, both aromatisable and non-aromatisable androgens stimulated progesterone production from developed follicles in intact animals (Lucky *et al.*, 1977). Similar action has been shown *in vitro* where androgens acted synergistically with FSH to stimulate progesterone production in granulosa cells (Armstrong & Dorrington, 1976; Nimrod, 1981). Therefore, androgens stimulate the side chain cleavage enzymes of cytochrome P450 and enhance the formation of the precursor substrates (progestins), from which they are formed.

The androgenic paracrine role changes during follicle development. *In vitro* studies using primate granulosa cells showed that the androgenic effect on FSH-induced ovarian function changed from being stimulatory to inhibitory as the pre-ovulatory development progressed (Harlow *et al.*, 1986; Harlow *et al.*, 1988). Hence, androgens seem to have bimodal paracrine functions during follicular development. Similar findings in the rat where the androgen receptor was also down regulated (Szoltys & Slomczynska, 2000) suggest that the mechanism of androgenic paracrine action involves androgen-induced down-regulation of the expression of the androgen receptor.

Since follicular fluid contains 5 α -reduced androgens, it is likely that some of the androgenic antagonistic effects are executed through the actions of DHT, androsterone and 5 α -A. McNatty *et al.* (1979b) showed that there were high levels of DHT in human ovaries. In fact, studies of the regulation of follicular maturation and the subsequent aromatisation of androgens in human (Hillier *et al.*, 1980b) and rat ovaries (Hillier *et al.*, 1980a) showed that 5 α -reduced androgens inhibited granulosa cell

aromatase activity *in vitro*. DHT reduced granulosa cell FSH-induced aromatisation of testosterone. Further evidence in the rat showed that naturally occurring 5α -androstane- $3\alpha,17\beta$ -diol and 5α -androstane- $3\beta,17\beta$ -diol inhibited FSH-stimulated secretion of oestradiol by granulosa cells even in the presence of exogenous testosterone, the precursor steroid from which oestradiol is metabolised (Ebong & Peddie, 1990). The evidence suggests that 5α -reduced androgens reduce oestrogen production by down-regulating aromatase activity.

5α -reduced androgens showed differences in their abilities to inhibit aromatase activity. Whereas 5α -A greatly inhibits aromatisation of its precursor, androstenedione, DHT does not similarly affect testosterone (Agarwal *et al.*, 1996). Of the reduced metabolites studied, 5α -A showed the greatest inhibition of oestradiol production (Ebong & Peddie, 1990; Agarwal *et al.*, 1996; Hutchison *et al.*, 1996). This steroid may, therefore, have an important intraovarian role in regulating follicle development due to direct inhibition of oestradiol secretion in maturing antral follicles.

Therefore, the paracrine role of ovarian androgens in regulating follicular development is dependent on the stage of follicular development. Generally, aromatisable androgens exert antagonistic actions on ovarian follicular development in preantral and early antral follicles, presumably to inhibit premature secretion of oestrogens. They switch to a facilitatory role on maturing follicles that have developed to the large antral stages of development, which require more substrates from which oestrogens are synthesised. However, the facilitation is transitory because the androgenic effect becomes inhibitory as pre-ovulatory development progresses (as discussed above). The dual role ensures that androgens contribute to the maintenance of immature small follicles at preantral stages until such a time when the ovary has attained reproductive maturity. Only then can follicles respond properly to the various stimuli among which the androgens contribute to the facilitatory mechanisms, which ensure that only mature follicles are prepared for ovulation.

The main role of the ovarian paracrine action is to regulate the balance between androgen output and conversion to oestrogens, so avoiding the premature secretion of excess androgens, which can impair follicle development.

1.4. OVARIAN METABOLISM OF ANDROGENS

In the ovary, androgens are principally present as intermediates in steroidogenic pathways, the main one being the synthesis of oestrogens (Hsueh *et al.*, 1984; Adashi, 1994). Minor pathways involve 5 α -reduction of androgens whose physiological roles are addressed later.

1.4.1. Aromatisation of Androgens

Androstenedione and testosterone are the main androgens in ovarian follicular fluid (McNatty *et al.*, 1979b). Androstenedione and to a much lesser extent testosterone, are synthesised by ovarian follicles throughout their most active phase of antral growth. They are aromatisable androgens and largely present as intermediates in the oestrogen biosynthetic pathway of the ovary (Hillier, 1981). This aromatisation reaction has been demonstrated *in vitro* in granulosa cells (Hillier *et al.*, 1980a; Wood *et al.*, 1994), whole follicles (Smyth *et al.*, 1994) and also *in vivo* (Amri *et al.*, 1993). Androstenedione and testosterone are aromatised into oestrone and oestradiol, respectively (Figs. 1.6 and 1.7). The ovarian aromatisation of androgens is a developmentally regulated process (Hsueh *et al.*, 1984; Tsafriiri *et al.*, 1994).

1.4.2. 5 α -Reductive Metabolism of Androgens

Androgens bearing a 4-ene-3-oxo-structure (like similar progestins) are metabolised by steroid 5 α -R1 into 5 α -reduced steroids (Gore-Langton & Armstrong, 1988: p337; Russell & Wilson, 1994). Some of the 5 α -reduced androgens that have been identified in various species are 5 α -dihydrotestosterone (DHT), 5 α -androstenedione (5 α -A), androsterone and epiandrosterone (Smith *et al.*, 1974; Gore-Langton & Armstrong, 1988). The presence of 5 α -androgens in follicular fluid (Khalil & Lawson, 1983; Dehennin *et al.*, 1987; Haning *et al.*, 1991; Anderson *et al.*, 1992; Jakimiuk *et al.*, 1999) indicates an alternative metabolic pathway (see Fig. 1.7) for androgens in the ovary. Therefore, 5 α -androgens are evidently produced in the ovary, but the function of 5 α -R is still unclear and has been addressed separately below.

1.5. 5 α -REDUCTASE

To date, the expression of 5 α -R in the ovary is indicated by the metabolism of 5 α -reduced androgens (as explained above) and other 5 α -reduced steroids (Karakawa *et al.*, 1976; Inaba *et al.*, 1978; McNatty *et al.*, 1979b) in follicular fluid. 5 α -R is a class of

microsomal enzymes that catalyse the saturation of the C-4,5 double bond of the A-ring of steroids Fig. 1.8; (Russell & Wilson, 1994). The biochemical step is stereospecific and requires NADPH which directly transfers a hydride ion to the carbon-5 position of the steroid (Abul-Hajj, 1972; Wang *et al.*, 1999). The enzymes are hydrophobic proteins and hence, extremely insoluble.

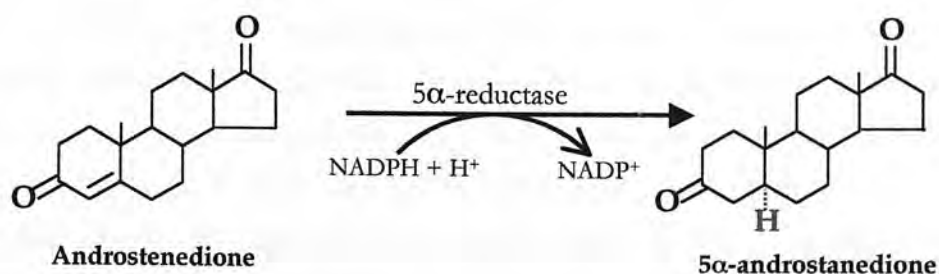


FIGURE 1.8. 5α-reductase reaction. Conversion of the double bond between carbons 4 and 5 of ring A of the steroid molecule is an NADPH-dependent reaction.

The enzyme exists in different species; including fish (Schlinger *et al.*, 1989), rodents (Normington & Russell, 1992; Russell & Wilson, 1994), and man (Russell & Wilson, 1994; Haning *et al.*, 1996). It also exists mainly as 2 isozymes, namely 5α-R type 1 and type 2, i.e., 5α-R1 and 5α-R2, respectively (Russell & Wilson, 1994). The amino acid sequences show that the type 2 isozymes of different species are more closely related to each other than to the type 1 isozymes. In fact, both isozymes are actually two different proteins encoded by the two different genes (Normington & Russell, 1992). As a result, the biochemical characteristics of the two types are distinguishable and can be used to identify them. For example, the type 1 isomers have neutral to basic pH optima while the type 2 isomers have acidic pH optima. The isozymes are also pharmacologically different, and are expressed differentially in reproductive and peripheral tissues (Russell & Wilson, 1994). The two isozymes have been described in the reproductive tissues of the human male (Andersson *et al.*, 1991) and female (Haning *et al.*, 1996), and also in the rat (Normington & Russell, 1992). The protein and gene structures of the rat and human forms also indicate that they are true homologues (Andersson & Russell, 1990; Russell *et al.*, 1994; Russell & Wilson, 1994) making the rat enzyme a good model for use in investigations aimed at studying the function and control of the human enzyme. Both the rat and human enzymes reduce steroids with the 3-oxo-Δ^{4,5} structure, such as progesterone, testosterone and androstenedione, and

have been shown to have preference of progesterone as substrate (Andersson & Russell, 1990).

1.5.1. Tissue Expression of 5 α -Reductase

5 α -reductase type 1 is present in various tissues; skin (Harris *et al.*, 1992), brain (Yokoi *et al.*, 1998), liver (Berman & Russell, 1993), and urogenital system (Berman *et al.*, 1995). Its expression is also present *in utero* (Berman *et al.*, 1995), where it is thought to control the formation of genitalia. Because 5 α -R is essential in male sexual differentiation, its expression in tissues has mostly been studied in relation to male physiology (Andersson & Russell, 1990; Habib *et al.*, 1998; Wright *et al.*, 1999; Soderstrom *et al.*, 2001), and its role in females, particularly, reproduction, is only emerging. The enzyme has very low levels of expression, even in androgen-responsive tissues, which has made its investigation difficult. In addition, its expression in female reproductive tissues such as the human ovary, is still controversial. While Haning *et al.*, (1996) identified only 5 α -R1 isozyme in the human ovary, Jakimiuk *et al.*, (1999) reported the expression of both 5 α -R1 and 5 α -R2 isozymes in the human ovary. The ovarian cellular expression of 5 α -R1 is yet to be specified because studies of the enzyme's activity suggest that it is located in both steroidogenic cell types of the ovary (Aono *et al.*, 1981; Payne *et al.*, 1992), and yet its activity is stimulated by LH in immature ovaries (Fukuda *et al.*, 1979), which suggests specific thecal expression.

1.5.2. Regulation of 5 α -Reductase Activity

Introductory studies on the activity of 5 α -R showed that it was controlled by LH (Fukuda *et al.*, 1979; Aono *et al.*, 1981), progestins (Eckstein *et al.*, 1970; Lerner & Eckstein, 1976; Payne *et al.*, 1992), as well as androgens (Smith *et al.*, 1974; Andersson & Russell, 1990). While the activity of the enzyme is higher in ovaries of immature rats compared to adults (Karakawa *et al.*, 1976), the intraovarian roles in either stage of development are still to be identified. Evidence in brain tissue suggests that 5 α -reductase activity begins during neonatal life and decreases with age and sexual maturation (Lephart & Ojeda, 1990). Although it is unclear whether a similar ontogeny is present in the ovary, some evidence suggests such a gonadal role whilst still *in utero* (Schindler, 1976).

In spite of the meagre information about the role of 5 α -R in female reproduction, particularly in relation to the production of 5 α -reduced androgens, it is clear that these steroids are inhibitors of oestrogen production as explained in section 1.5.3. It is possible that the elevated levels of 5 α -reduced androgens in PCOS exacerbate the arrested follicle development characteristic of polycystic ovaries as postulated by Jakimiuk *et al.*, (1999).

1.5.3. Physiological Role of 5 α -reduced Androgens

Although 5 α -reduced androgens have been identified as a major component in follicular fluid, studies of their roles in female physiology are still largely inconclusive. Specifically, there is not much information about the role of 5 α -reduced androgens in ovarian function. However, it has been shown that 5 α -reductive metabolism is an age-related biosynthetic step. Immature rat ovaries secreted large amounts of 5 α -androstane-3 α ,17 β -diol unlike adult rats (Eckstein *et al.*, 1970; Karakawa *et al.*, 1976). It has been suggested that 5 α -reduced androgens have an important role in controlling the onset of puberty (Ebong & Peddie, 1990). However, other work failed to support the relationship between 5 α -reduced androgen production and puberty (Ojeda *et al.*, 1984).

The role(s) of 5 α -reduced androgens in female reproduction is/are difficult to compare with earlier studies which mostly focussed on male fertility where they are crucial for development of secondary sexual characteristics (Booth, 1977; Andersson *et al.*, 1991; O'Donnell *et al.*, 1996). It is clear though that until puberty, the major metabolites of progesterone in immature female rats, unlike the adults were 5 α -reduced progestins (Eckstein *et al.*, 1970; Lerner & Eckstein, 1976) and 5 α -reduced androgens (Eckstein *et al.*, 1970; Karakawa *et al.*, 1976). Thus, the steroidogenic activity of the immature rat gonad is considerably different from that of the adult. Specifically, the steroidogenic pathways in ovaries of immature rats lead to the formation of 5 α -androstane-3 α ,17 β -diol via 5 α -reduced progestins while the pathways in adult ovaries form oestrogens via the Δ^4 -steroids.

5 α -reduced androgens cannot be aromatised and, therefore, do not have a substantive role in oestrogen synthesis. In addition, naturally occurring 5 α -reduced

androgens, DHT and 5 α -A, competitively inhibited granulosa cell aromatase action *in vitro* (Hillier *et al.*, 1980a) suggesting an intraovarian mechanism for inhibition of oestrogen synthesis. It has also been demonstrated that they inhibit gonadotrophin secretion in rats (Zanisi *et al.*, 1973). It is possible that the role of these androgens is to suppress development-related production of oestrogen in immature animals because lack of oestrogen contributes to the non-developmental state of prepubertal follicles. Interestingly, 5 α -androstanediol, a metabolite that is produced in immature rats causes vaginal opening in rats (Eckstein *et al.*, 1970) suggesting as yet unclarified roles of 5 α -reduced androgens in female physiology.

Thus, 5 α -reduced androgens act at the hypothalamic and ovarian levels as part of the mechanisms that inhibit oestrogen production. Although not clear, the role of 5 α -reduced androgens is probably related to the inhibition of oestrogens in immature animals that have not yet attained puberty. In doing so, these androgens probably contribute to the intraovarian environment, which ensure that primordial follicle development does not start until such a time when adult changes in the ovary ensure that oestrogen production will be in synchrony with follicle development and support normal pre-ovulatory development that can result in reproduction.

1.6. POLYCYSTIC OVARY SYNDROME

Polycystic ovarian syndrome (PCOS) is a very common endocrine disorder in women, ranging 10-20% in different populations and is the major cause of anovulatory infertility (Franks, 1997; Knochenhauer *et al.*, 1998; Rodin *et al.*, 1998; Franks *et al.*, 1999a; Dunaif & Thomas, 2001). It is a classic example of the consequences of dysfunctional follicle development and steroid biosynthesis. However, the condition has a very broad spectrum of clinical and biochemical presentations, which confound studies of its aetiology. Clinically, the women present with hirsutism, oligomenorrhea, chronic anovulation, and hence infertility, obesity and morphological changes of the ovaries. The biochemical abnormalities are elevated testosterone, androstenedione, LH, oestrone, oestradiol levels, increased LH:FSH ratio, hyperinsulinism and reduced concentration of sex hormone binding globulin (SHBG) (Speroff *et al.*, 1983; Conway *et al.*, 1989; Franks, 1989; Rebar, 1995; Dewailly, 1997).

Generally, diagnosis of PCOS is made when the typical ultrasound features of polycystic ovaries are present with oligo/amenorrhoea and/or clinical symptoms of hyperandrogenism such as hirsutism or acne without specific underlying diseases of the adrenal or pituitary glands, (Homburg, 1996). Polycystic ovary diagnosis is based on the presence of more than 8 discrete follicles of less than 10 mm diameter (cysts) in the ovary, usually peripherally arrayed around an enlarged, central stroma which occupies more than 25% of the ovarian volume (Homburg, 1996; Dunaif & Thomas, 2001). The ovaries are usually, but not always enlarged. In fact, the clinical picture of women with PCOS does not seem to vary with change in ovarian size and so, there is seemingly no basis for classification of polycystic ovaries into those of normal or greater than normal size (Goldzieher & Young, 1992).

Although the morphological identification of polycystic ovaries by ultrasound is the basis of PCOS diagnoses, but by itself, is not definitive of the syndrome because polycystic ovaries are found in normal women (Polson *et al.*, 1988; Clayton *et al.*, 1992; Farquhar *et al.*, 1994; Knochenhauer *et al.*, 1998; Koivunen *et al.*, 1999) and in other endocrine disorders such as adrenal 21-hydroxylase deficiency (Abdel Gadir *et al.*, 1992a). Therefore, the presence of polycystic ovaries must be supplemented with an endocrine biochemical disorder to diagnose PCOS (Swanson *et al.*, 1981; Polson *et al.*, 1988; Franks, 1989; Abdel Gadir *et al.*, 1992b). Generally, PCOS is a syndrome that is clinically associated with hyperandrogenism, anovulation and metabolic disorders.

1.6.1. Follicle Development in PCOS

The cause of the abnormal follicle development in women with PCOS is still unknown. The characteristic "cysts" are not atrophic, apoptotic or fibroid tissue, but follicles whose development was held-in-check when they had attained a size of 6-8mm in diameter (Fauser, 1994; Takayama *et al.*, 1996). They are still steroidogenically robust and can undergo further growth and development when supplied with adequate endocrine stimulation (Takayama *et al.*, 1996; Anderson *et al.*, 1997).

It was also shown that the anomaly in PCOS does not seem to result from abnormalities in the hormone primarily responsible for follicle growth, i.e., FSH. Circulating immunoreactive FSH levels (Van Dessel *et al.*, 1995) as well as bioactive FSH serum levels (Fauser *et al.*, 1991) and follicular fluid levels (Erickson *et al.*, 1992) in

anovulatory women with PCOS are within normal preovulatory range. In addition, the granulosa cells from polycystic ovaries showed normal or even elevated FSH-induced aromatase activity *in vitro* (Speroff *et al.*, 1983). Since FSH stimulation and granulosa aromatase activity per se seemed to be within normal limits, the arrested follicle growth observed in PCOS is probably due to altered intra-ovarian regulation of FSH-induced actions. It is possible that locally produced ovarian factors such as cytokines (Homburg & Amsterdam, 1998), cyclic AMP (Franks *et al.*, 1998) or steroids (Mason & Franks, 1997) are involved in the atypical follicle development.

As in normal follicles, developmental progress to the size of follicles noticed in polycystic ovaries occurs without an associated increase in oestrogen biosynthesis (Hillier, 1994a), but further development of the follicles that is "arrested" in these ovaries. The cause of this "arrest" is still controversial. Various hypotheses have suggested hypothalamic-pituitary origins (Weisz & Lloyd, 1965), intraovarian origins (Gilling-Smith *et al.*, 1997; Dewailly, 1999; Rosenfield, 1999; Franks *et al.*, 2000), genetic factors (Franks *et al.*, 1999b), altered insulin metabolism (Panidis *et al.*, 1997; Dewailly, 1999), and nutritional and environmental factors (Crosignani & Nicolosi, 2001) as the cause of PCOS. The causal debate of PCOS still continues and it is more likely that the syndrome arises as a result of a combination of some of these factors. Whatever the cause of PCOS, the major consequence of the altered ovarian function is the creation of an intraovarian environment that is inhibitory to further follicle development, the result being the formation of increased numbers of pre-antral follicles (cysts) and stromal hyperplasia, the two main morphological features of polycystic ovaries.

As already mentioned, abnormal endocrine profiles are also a feature of PCOS. The syndrome is associated with disorders of gonadotrophin secretion, acyclic oestrogen production, hyperandrogenism and hyperinsulinaemia (Marshall, 2001). There is no single biochemical marker of PCOS. Confounding still, hyperandrogenism together with insulin resistance are common features of other aetiologies other than PCOS (Goldzieher & Young, 1992). However, elevated levels of serum androgens are a consistent symptom in all women with the syndrome's typical ovarian morphology (Rodin *et al.*, 1994; Rebar, 1995; Homburg, 1996).

1.6.2. Hyperandrogenaemia in PCOS

The characteristic steroidogenic abnormality of PCOS is increased androgen production (Speroff *et al.*, 1983; Franks & White, 1993; Rodin *et al.*, 1994). In particular, elevated testosterone and androstenedione concentrations are the most common abnormal hormonal features of the syndrome (Conway *et al.*, 1989; Franks, 1989). However, there is controversy over the source of the excess androgen secretion. Evidence that both androgen-producing organs, the ovaries and adrenals (Fruzzetti *et al.*, 1995; Rosenfield, 1999; Dunaif & Thomas, 2001) contribute to the hyperandrogenemia, lies alongside that which argues for (Rodin *et al.*, 1994; Azziz *et al.*, 1998; Moran & Azziz, 2001) and against (Wajchenberg *et al.*, 1986) an adrenal contribution. Direct measurements of androgens in blood samples from left adrenal and left ovarian veins suggested that the ovary is the predominant source of the excess androgen observed in PCOS (Wajchenberg *et al.*, 1986).

Functional ovarian hyperandrogenism is found in 70% of women with PCOS (Franks & White, 1993; Rosenfield, 1999). The thecal -interstitial compartment, due to exaggerated enzyme(s) activity produces excess steroids, particularly androgens. Immunohistochemical studies of polycystic ovaries showed that the small follicles (≤ 7 mm in diameter) had increased expression of P45017 α lyase (Tamura *et al.*, 1993) indicating that the hyperandrogenemia in PCOS was definitely related to upregulation of the androgen-synthesising enzymes. The dysregulation of P450c17 α within ovarian thecal cells, either by excessive stimulation by LH or by escape from desensitization to LH was indicated as a cause of the hyperandrogenemia (Rosenfield *et al.*, 1990). The excessively increased enzyme action in PCOS, also reported by others (Qin & Rosenfield, 1998) was thought to result from an intrinsic intraovarian flaw in the paracrine feedback mechanism by which thecal androgen biosynthesis is inhibited.

Investigations using activity assays indicated specifically that thecal cells from PCOS follicles secreted increased amounts of androstenedione, and 17-hydroxyprogesterone, an intermediate steroid between the androgen and glucocorticoid biosynthetic pathways (Fig. 1.6; Gilling-Smith *et al.*, 1994). The increased conversion of progesterone to androstenedione in the thecal of polycystic ovaries was independent of the ovulatory status. The ovarian follicles from women with PCOS were also seen to have small numbers of granulosa cells and hardly expressed aromatase (Tamura *et al.*,

1993). Therefore, whether due to increased androgen production and/or decreased androgen metabolism, hyperandrogenemia in PCOS is basically a condition resulting from dysfunctional ovarian steroidogenesis.

There is also a correlation between increased stromal size and overproduction of thecal -derived steroids, particularly, androstenedione (Kyei-Mensah *et al.*, 1998). In light of this and the increased expression of the enzyme, 5 α -R in polycystic ovaries (Jakimiuk *et al.*, 1999), it is plausible that androstenedione is metabolised to 5 α -A, and that the androgenic effects in PCOS are partly enhanced by the formation of 5 α -reduced androgens. Measurements of 5 α -androstadiol glucuronide, a metabolite showing 5 α -R activity in peripheral tissues, indicated that serum concentrations of the enzyme were higher in women with PCOS than among normal controls (Franks, 1989) suggesting increased activity of the enzyme in women with PCOS. In fact, 5 α -A was shown to be greatly increased in follicular fluid from polycystic ovaries compared to normal follicular fluid (Agarwal *et al.*, 1996). Being a competitive inhibitor of aromatase (Hillier *et al.*, 1980a), 5 α -A is probably one of the ovarian steroids that modulate ovarian processes locally. It is thought that the increased levels of 5 α -A in polycystic ovaries, by decreasing aromatase activity, result in decreased oestradiol production, which in turn hinders further growth and development of the ovarian follicles (Agarwal *et al.*, 1996). This mechanism suggested an intraovarian system in PCOS that acted on itself to amplify the formation of polycystic ovaries. Undoubtedly, the lack of adequate levels of oestrogen in polycystic ovaries contributes to maintain the antral follicles in the arrested state since dominance is dependent on a surge in ovarian oestrogen production.

High levels of androgens become a problem when they are available to tissues as biologically active steroids. The physiological effects of androgens are controlled by their availability, which is partly dependent on the proteins that transport them around the body. Androgens are mainly carried in blood bound to SHBG, and some are carried by albumin. Androgens carried by SHBG are unavailable for bio-action, but albumin-bound steroids are readily available. The latter and "free" androgen (unbound to SHBG) are responsible for the androgenic physiological responses. Androgenic effects can also be enhanced by further metabolism into more potent forms, for example,

5 α -reduced androgens that are generally more potent than aromatisable androgens in target tissues.

1.6.3. 5 α -Reductase in PCOS

Although the role(s) of 5 α -reduced androgens are still unclear in the female, 5 α -R is evidently up-regulated in PCOS as shown by the increased ovarian 5 α -R activity (Jakimiuk *et al.*, 1999), and a high ratio of 5 α - to 5 β -cortisol metabolites in the urine of women with PCOS (Stewart *et al.*, 1990). Women with polycystic ovaries also had increased peripheral conversion of testosterone and androstenedione to the more potent 5 α -reduced forms (Franks, 1989). Earlier work by Forleo *et al.* (1971) showed that stromal tissue and the "cysts" in wedge resections of ovarian tissue from polycystic ovaries had 5 α -R activity, and could metabolise 5 α -A and androsterone *in vitro*. Clearly, 5 α -R activity is elevated and functional in PCOS where its metabolites probably aggravate the abnormal follicle development and steroidogenic patterns which are potential causes of infertility.

1.7. SUMMARY OF THESIS OBJECTIVES:

To investigate further the role of 5 α -reduced androgens in the paracrine control of ovarian function, the use of an animal model was adopted complementing observations on human ovaries. The aims were (1) to establish the extent to which individual cell types in the rat ovary metabolise androgens to 5 α -reduced androgens and oestrogens, respectively, (2) to study the location and developmental regulation of 5 α -R1 gene expression in the rat ovary, and (3) also to obtain evidence for increased formation of 5 α -reduced androgens in polycystic ovaries.

Chapter 2. Materials

Animals

Immature and mature female rats of the Wistar strain were obtained from Charles Rivers (Margate, Kent, UK) and housed in a temperature and light-controlled room. All animals used in experimental procedures were killed by carbon dioxide asphyxiation.

Antisera

5 α -Reductase-1 antibody used in immunocytochemical studies was donated by Dr. Gerhard Aumuller, (Department of Anatomy and Cell Biology, Philipps-University Marburg, Germany). Antiserum used in the 17KA RIA was rabbit anti-androsterone-3-hemisuccinyl-BSA. Major crossreactivities were: 5 α -androstane-3,17-dione, 100%; androstenedione, 70%; androsterone, 63.6%; and less than 0.5% for all other steroids tested. Androstenedione RIA used rabbit anti-androst-4-ene-3,17-dione-7 α -carboxyethyl thioether conjugated to BSA. Major crossreactivities were: androstenedione, 100%; androsterone, 46.3%; 5 α -androstene-3,17-dione, 50%; testosterone, 37%; and less than 0.5% for all other steroids tested. Oestradiol RIAs were performed using rabbit anti-oestradiol-6-(O-carboxymethyl)-oximino-BSA serum with crossreactivities: 17 β -oestradiol, 100%; oestrone, 2.3% and less than 0.1% for all other steroids tested.

Chromatography sheets

20 x 20 cm aluminium oxide- and silica-coated TLC sheets with and without UV₂₅₄ detection were obtained from CamLab Ltd., Cambridge, UK.

Enzymes

All restriction endonucleases and DNA modifying enzymes were obtained from Promega Corporation, Southampton, UK.

Fixatives

Bouins fixative was obtained from Triangle Biomedical Sciences (Skernersdale, UK) and normal buffered formaldehyde (NBF) was prepared with reagents obtained

from BDH, Lutterworth, Leicester, UK: 100 ml 40% formaldehyde v/v, 4g sodium dihydrogen phosphate monohydrate and 6.5g disodium hydrogen phosphate anhydrous dissolved in 1 L distilled water.

Follicular fluids

Follicular fluids from normal women and women with PCOS were obtained from follicles at different stages of development as described by Mason et al., (1994). Samples were donated by Professor Steve Franks (Department of Reproductive Science and Medicine, Imperial College School of Medicine, London, UK).

Gene Cloning

Gene cloning reagents were obtained from Stratagene, Cambridge, UK. Unless stated otherwise, plasmid DNA purification systems were obtained from Qiagen Ltd., (UK). Gene sequencing was performed with ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit obtained from PE Applied Biosystems, Warrington, Cheshire, UK.

Histology

All the reagents used for *in situ* hybridisation (ISH) were obtained from Sigma/Aldrich Ltd. (Poole, Dorset, England) unless mentioned otherwise.

Hormones

Equine CG (2000 IU eCG/mg) and hCG (14,000 IU LH/mg) were obtained from Sigma/Aldrich Ltd. Working concentrations were 100 IU/ml PBS (tissue culture grade). Recombinant human FSH (3860 IU FSH/mg) and recombinant human LH (17,500 LH IU/mg) were donated by Dr. Howles (Serono Laboratories, Inc., Welwyn Garden City, UK). Concentrations of stock solutions were 10 ng/ml in PBS.

Nucleic acid markers

1 Kb DNA ladder (Cat. No. 15615-016) was obtained from GibcoBRL, Life Technologies Ltd., Paisley, UK.

Nucleic acid probes

Complementary RNA probes were synthesised with MAXIscript™ *in vitro* transcription kit (Ambion, Cat. No. 1324) or Strip-EZ RNA™ StripAble™ RNA probe synthesis and removal kit (Ambion, Cat. No. 1368) obtained from A.M.S. (UK) Biotechnology, Abingdon, Oxon, UK. Complementary DNA probes were synthesised with Rediprime™II random prime labelling system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The synthesised probes were purified with MicroSpin™ G-50 columns (Amersham Pharmacia Biotech, Cat. No. 27-5330).

Oligonucleotides

5 α -R1 and aromatase primer oligonucleotides were obtained from Cruachem Ltd., Glasgow, UK.

RNA analyses

Unless stated otherwise, reagents, buffers, enzymes used for RNA analyses were obtained from A.M.S. (UK) Biotechnology. RNazol B was bought from Tel-Test, Inc. (Friendswood, USA). Hybond-N nylon hybridisation transfer membrane was supplied by Amersham Pharmacia Biotech (Cat. No. RPN 203 N) was used for RNA blotting.

Solvents

HPLC grade ethanol and diethyl ether were obtained from BDH. All other reagents, unless mentioned otherwise were obtained from Sigma/Aldrich Ltd.

Steroids

All the non-radiolabelled steroids were obtained from Sigma/Aldrich Ltd. The steroids were stored as stock solutions of 1 mg/ml in ethanol at 4°C. With the exception of tritiated androsterone, all the radioactive steroids were purchased from Amersham Pharmacia Biotech. Tritiated androsterone was purchased from NEN™ Life Science, Hounslow, UK. The radiolabelled steroids were stored as stock dilutions (1:10) in 100% HPLC grade ethanol at 4°C. Radiolabelled nucleotides were obtained from Amersham Pharmacia Biotech and stored at -70°C. The specific activities of the radiolabelled compounds are listed in Table 2.1.

TABLE 2.1. Radiolabelled steroids

Steroid	Preparatory solvent	Specific activity
[9,11- ³ H(N)]androsterone (³ H-androsterone)	ethanol	1998.0 GBq/mmol 54.0 Ci/mmol
[1,2,6,7- ³ H]androst-4-ene-3.17β-dione (³ H-androstenedione)	toluene:ethanol solution (9:1)	3.74 TBq/mmol 101 Ci/mmol
[2,4,6,7- ³ H]oestradiol (³ H-oestradiol)	toluene:ethanol (9:1)	3.03 TBq/mmol 82.0 Ci/mmol
5α-Dihydro[1,2,6,7- ³ H]testosterone (³ H-5α-dihydrotestosterone)	toluene:ethanol solution (9:1)	3.59 TBq/mmol 111Ci/mmol
[1,2,6,7- ³ H]testosterone (³ H-testosterone)	toluene:ethanol solution (9:1)	3.59 TBq/mmol 97.0 Ci/mmol
[1,2,6,7- ³ H]progesterone (³ H-progesterone)	toluene	3.18 TBq/ mmol 86.0 Ci/mmol
Molecular Biology Reagents		
[³⁵ S]UTP	aqueous solution	>1000 Ci/mmol 20 mCi/ml
[α- ³² P]UTP	aqueous solution	800Ci/mmol 20 mCi/ml
Redivue [α- ³² P]CTP	aqueous solution	~3000 Ci/mmol 10 mCi/ml

Tissue culture

Antibiotics, enzymes and buffer constituents used in tissue culture studies were obtained from Sigma/Aldrich Ltd., (Dorset, England).

Chapter 3. Gonadotrophic regulation of ovarian androgen metabolism

3.1. INTRODUCTION

Androgen metabolism, as explained in Chapter 1, is central to ovarian steroidogenesis and follicle growth during the antral stages of follicle development that culminate in ovulation. Androstenedione, the principal aromatisable androgen in human and rat ovaries (among other species) for oestrogen synthesis (Gore-Langton & Armstrong, 1988), was used as the androgenic substrate in this study's investigation of androgen metabolism in the ovary. In addition, the implication that hyperandrogenaemia of PCOS was partly due to androstenedione metabolism via its 5α -reduced metabolite, 5α -A, made androstenedione the androgen of choice when investigating androgen metabolism in the ovary. The presence and metabolism of high levels of androstenedione in rat follicular fluid indicate that androstenedione metabolism is a normal physiological process in rat ovary (Karakawa *et al.*, 1976; Fujii *et al.*, 1983; Anderson *et al.*, 1992), thereby making the rat an ideal animal model for studying androstenedione metabolism.

In normal pre-ovulatory follicles, androstenedione is mainly metabolised to oestradiol via two principal pathways. It is converted to either oestrone or testosterone by the enzymes aromatase and 17β HSD, respectively (see Chapter 1, Fig. 1.2). An alternative minor metabolic pathway catalysed by the steroidogenic enzyme, 5α -R converts androstenedione to 5α -A. Steroid 5α -R, a membrane bound enzyme, converts 3-oxo- $\Delta^{4,5}$ unsaturated steroids into 5α -reduced metabolites with altered endocrine potential (Russell & Wilson, 1994).

The roles of gonadotrophins, FSH and LH in regulating aromatisable ovarian androgen synthesis, which precedes oestrogen production, are well documented. In essence, LH selectively stimulates androgen production (Erickson *et al.*, 1985) while FSH stimulates the bio-conversion of androgens to oestrogens (Hillier, 1985). However, the roles of gonadotrophins in the synthesis/metabolism of 5α -reduced androgens are

less clearly defined. Inconclusive studies of the gonadotrophic effects on the production of 5 α -reduced androgens have led to uncertainties over their roles (Armstrong & Papkoff, 1976; Karakawa *et al.*, 1976) in relation to disease processes (Agarwal *et al.*, 1996; Jakimiuk *et al.*, 1999), and in normal adult ovarian cycles (Lephart *et al.*, 1992). Studies of 5 α -R and gene expression are also incomplete (Jakimiuk *et al.*, 1999).

To investigate the role of gonadotrophins in the biosynthesis of 5 α -reduced androgens, the production of 5 α -reduced androgens from androstenedione metabolism was investigated under conditions that reflected pre-ovulatory follicular development (see Chapter 1). Using *in vivo* and *in vitro* experimental strategies, androstenedione metabolism was investigated in conditions that favoured or inhibited oestrogen production. Since the ovary is enzymically designed to synthesise oestrogens from androgens (Hillier, 1985; Richards, 1994), blocking oestrogen production should steer the steroidogenic ability to alternative pathways such as the 5 α -reductive pathway because the androstenedione substrate is not then converted into oestrogens. Inhibition of oestrogen production was effected by Arimidex (Anastrozole), a highly potent and selective non-steroidal inhibitor of aromatase, which has no detectable pharmacologic activity aside from aromatase inhibition (Plourde *et al.*, 1994; Dukes *et al.*, 1996). Of particular interest to this study, Arimidex has no discernible effects on other steroid biosyntheses, making it an ideal compound to inhibit aromatase specifically.

Follicular development was induced in immature female rats *in vivo* by equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG). In all the studies, 21 day old rats were used for the treatments because at this age, ovulation has not begun, but their ovaries are morphologically and histochemically well developed (Kaczkowska & Liebhart, 1984). Equine CG, although a composite gonadotrophin in the mare, acts principally as an FSH in many other species including rats (Hoppen, 1994) and was therefore used to induce follicle growth. Human CG, bearing LH activity was used to induce ovulatory changes. This chapter describes a study designed to determine the androgen metabolic profile during eCG and hCG stimulated ovarian developments, using granulosa and thecal/interstitial cell culture systems. Most importantly, the relative influence of aromatisation versus 5 α -reduction was investigated.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. Animals

Intact immature female rats were injected once subcutaneously (s.c) with 10 IU eCG followed 48 h later by a single injection of hCG (s.c), and the removal of ovaries 12 h or 24 h later. Control animals received no hormonal treatment. Sham-treated controls were not used since pilot experiments showed no non-specific response at the ovarian level. The outline of the treatment protocol is illustrated in Fig. 3.1. All the animals were killed by carbon dioxide asphyxiation on the same day after completion of the hormonal treatments.

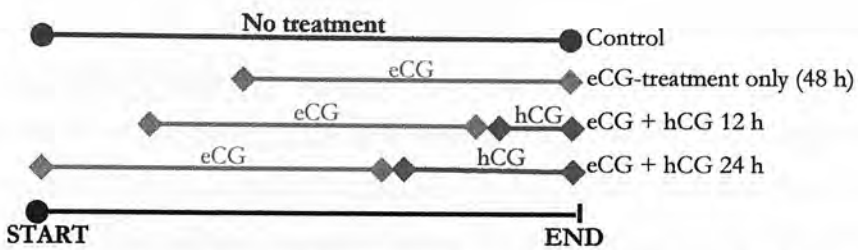


FIGURE 3.1. Outline of hormonal treatment regimen administered to induce preovulatory follicle growth. Treatments were started on different days so that all the tissues could be harvested on the same day.

3.2.2. Isolation of Granulosa Cells

Ovaries were harvested and immediately placed in medium M199 (M199) supplemented with L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). Using stereomicroscopic visualisation, fat surrounding the ovary and associated structures such as attached oviducts were removed. The ovaries were then transferred into fresh culture medium, for isolation of granulosa cells. Viewed by the stereomicroscope, all visible follicles were punctured using a 27G hypodermic syringe needle to release granulosa cells. The cell clumps were then combined in fresh M199 and resuspended by gentle, repeated pipetting. The suspension was transferred to a 12 ml conical centrifuge tube and gravity sedimented for 5 min so that cellular debris could settle at the bottom of the tube. Granulosa cells remaining suspended in medium were pipetted into a fresh centrifuge tube in which they were centrifuged at 200 g for 10 min. The cell pellet was gently re-suspended in 10 ml fresh M199 and aliquots of this cell suspension were used to set up granulosa cell cultures, after determination of viable cell number.

Cell counts were performed using a haemocytometer. An aliquot of 20 μ l of the cell suspension was mixed with an equal volume of 0.4% trypan blue dye, which stains dead cells blue because the ruptured cell membranes allow the dye to penetrate the cytoplasm while viable cells appear colourless. After counting the numbers of dead and viable cells, cell counts were corrected for the various factors to obtain total and viable cell numbers. Cell viability estimated this way was typically 20% (n=4).

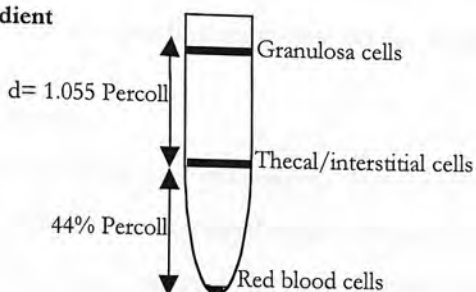
3.2.3 Isolation and Culture of Thecal/Interstitial Cells.

Thecal/interstitial cells were harvested from residual ovary after removal of granulosa cells as explained above. Residual ovarian tissue was rinsed in M199 and resuspended in 5 ml fresh M199 containing 0.1% (w/v) collagenase *Cl. histolyticum* type II and 0.01% DNase for enzymic digestion at 37°C for 30 min in a shaking waterbath. Complete dispersal into a single-cell suspension was achieved by repeatedly pipetting the digest at the end of the incubation. The cells were then collected by centrifugation at 800 g for 5 min and resuspended in 2 ml fresh M199. At this stage, the thecal/interstitial cell preparations were contaminated with granulosa cells, which were removed by discontinuous density centrifugation (Magoffin & Erickson, 1988). The thecal/interstitial cell fraction was purified by separating the cell mixture through a centrifugation ladder created by layering two Percoll solutions of different specific gravities prepared in M199 (Fig. 3.2). Two ml of a Percoll solution of specific gravity 1.055 were layered on top of 1 ml of 44% Percoll solution. The suspension of dispersed thecal/interstitial cells was carefully layered on top of the d=1.055 Percoll layer. The tubes were then centrifuged at 400 g for 20 min, by which time the cell types were separated as in Fig. 3.2. Thecal/interstitial cells were removed by aspiration from the d=1.055 layer, washed in 5ml fresh M199 and centrifuged at 800 g for 5 min. After removal of the wash supernatant, the cell pellet was resuspended in 2 ml fresh medium containing 5.0% donor calf serum. An aliquot of 20 μ l of the suspension was used to determine the viable cell count. Cell viability, determined by staining with trypan blue was consistently greater than 90%.

3.2.4. Whole Ovaries

Whole ovaries from immature rats were put on dry ice immediately after removal and stored at -70°C until use. (5 α -reductase activity is stable for at least 3 months at this temperature (Payne *et al.*, 1992).

A. Percoll gradient



B. Formula

$$V_o = V \left[\frac{d - 0.1dx - 0.9}{d_o - 1} \right]$$

V_o = volume of Percoll (ml)

d_o = specific gravity of Percoll (1.130 g/ml)

dx = specific gravity of M199 (1.058 g/ml)

V = volume required for final working solution (ml)

d = specific gravity required for final working solution (g/ml)

FIGURE 3.2. Purification of ovarian thecal/interstitial cells by discontinuous Percoll centrifugation. Dispersed residual ovarian tissue was centrifuged through a Percoll gradient (A). Percoll solutions of specific gravity $d = 1.055$ were obtained using indicated formula (B).

3.2.5. Cell Cultures for Studies of Androstenedione Metabolism

3.2.5.1. Cell cultures of *in vivo* gonadotrophin-stimulated ovaries

Granulosa and thecal/interstitial cells from ovaries of rats stimulated *in vivo* as described in section 3.2.1 were used to set up cell cultures as follows.

I. Granulosa cell cultures

Except as noted, replicate 250 μ l aliquots of M199 containing 1×10^4 viable granulosa cells were pipetted into 24-well (16mm) polystyrene culture dishes that had been pre-coated with donor calf serum (DCS) for 24 h (Hillier and de Zwart, 1982). The incubate was adjusted to a final volume of 500 μ l by addition of 200 μ l non-radioactive androstenedione (final concentration 1.8 μ M), and 50 μ l Arimidex (6.1 μ M) in culture medium. 'Blank' medium replaced Arimidex solution in cultures lacking the aromatase inhibitor. Parallel cultures containing a trace amount of 5 μ l tritiated androstenedione (1×10^5 cpm; final specific activity- 9.2×10^{-7} μ Ci/mmol), were set up to study the formation of 3 H-labelled androstenedione metabolites. The cell cultures were incubated for 24 h at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air. At the end of the incubation, the culture medium was aspirated and pooled into 12 x 75 polystyrene test tubes, which were stored at -20°C until analysis. Thawed 0.5 ml

aliquots of culture medium were extracted with 4.0 ml diethyl ether for analysis of steroid metabolism by RIA or radiochromatography as described below.

II. Thecal cell cultures

Replicate thecal/interstitial cell cultures were set up at a cell density of $0.5 - 2 \times 10^5$ cells per 500 μ l M199 and incubated under similar conditions to granulosa cells in the presence of [3 H]androstenedione ($\sim 3.1 \times 10^{-4}$ nmols; final specific activity- 9.2×10^{-7} μ Ci/mmol).

3.2.5.2. Cell cultures of thecal/interstitial cells stimulated *in vitro*

To study gonadotrophin action *in vitro*, thecal/interstitial cells from untreated immature ovaries, were incubated in triplicate in 12-well (32 mm) DCS-coated polystyrene tissue culture dishes at a cell density of 1×10^5 viable cells per 1.0 ml M199 containing androstenedione (30 μ M), in the absence or presence of LH (3 ng/ml). The cultures were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air for 24-96 h. At the end of the culture period, medium was collected and stored at -20°C until analysis by RIA. The cell monolayers were washed with fresh serum-free medium followed by the addition of fresh M199 containing 3 H-labelled steroid substrate (1×10^5 dpm; 9.2×10^{-7} μ Ci/mmol) for 24 h. The medium was then collected and stored at -20°C until analysis by radiochromatography.

3.2.6. 5 α -Reductase Assay

Except as noted, whole ovaries (~ 2 mg wet weight whole ovary per ml) or viable thecal/interstitial cells (1×10^5 per ml) from immature rats were homogenised in assay buffer on ice using a manual glass/glass (Wheaton, USA) homogeniser. The assay buffer contained 50 mM potassium phosphate, 1 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol and 0.25 M sucrose at pH 7.4. Various tritiated steroid substrates (1×10^5 dpm), added together with corresponding non-radiolabelled steroids to give the required specific activity, were added to the tissue homogenates. The reactions were started by adding NADPH to a final concentration of 1 mM in a total reaction volume of 1 ml. Following incubation at 37°C for 0.5 to 2 h in a shaking waterbath, the reactions were stopped by transferring the tubes to a -20°C refrigerator where they were stored. The steroid metabolites were extracted with 4.0 ml diethyl ether and detected by radiochromatography.

3.2.7. Ether Extraction of Steroids

Incubation medium (0.5 ml from cell cultures) or reaction buffer (1.0 ml from 5 α -reductase assay) was transferred to 16 x 100 mm glass test tubes to which 4.0 ml diethyl ether was added. The tubes were mixed vigorously on a multi-rack electric mixer (S/P Multitube vortexer, Baxter Scientific) at high speed (speed number 5) for 1 min and left to stand until separation of the aqueous and organic phases. The upper organic phase, containing steroids was decanted into fresh tubes after snap freezing the contents of the extraction tubes on a mixture of solid CO₂ pellets in alcohol. The organic phase was then evaporated at 55°C under a stream of N₂ gas. The dry residues were then available for analysis by radiochromatography.

3.2.8. Analysis of Androstenedione Metabolites

3.2.8.1 Thin-layer radiochromatography of androstenedione metabolites

Dry steroid residues recovered by ether extraction were redissolved in 3 drops of acetone for separation by TLC. Using a 10 μ l pipette, each steroid solution was "spotted" at a pre-marked position on the origin of a demarcated 20 x 20cm Polygram aluminium oxide (alumina)-pre-coated TLC sheet containing UV₂₅₄ detection (Macherey-Nagel, Germany; Fig. 3.3) with evaporation under a stream of hot air. The dissolution in acetone and spotting were repeated to ensure thorough transfer of all extracted steroids onto the TLC sheets. Loaded TLC sheets were developed twice with cyclohexane:toluene: ethanol (100:95:5 v/v/v) as solvent system in pre-saturated 27.0 x 26.5 x 7.0cm glass tanks (Sigma-Aldrich) lined with Whatman 3mm paper. The

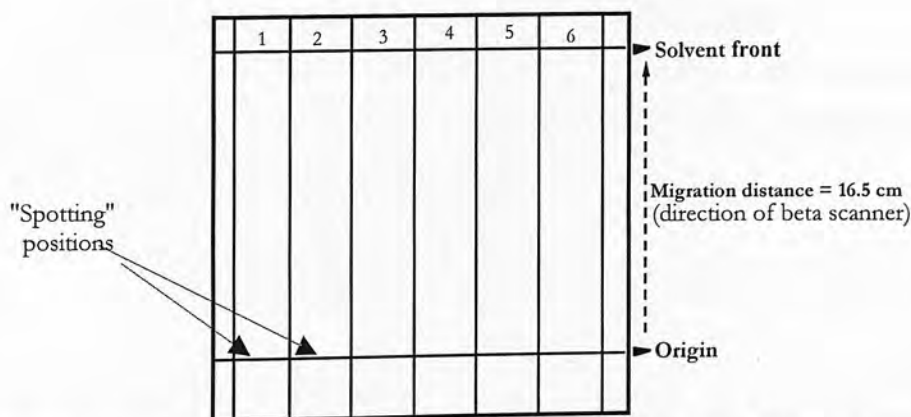


FIGURE 3.3. TLC sheet. The sheets were demarcated into lanes for easy spotting of sample and identification of spots of separated steroids. Loading was done in spots less than 3 mm in diameter to ensure narrower peaks and more distinct separation of steroids.

developed TLC sheets were air-dried, and the tritiated metabolites located using a beta emission scanner (Bioscan System 200 Imaging Scanner-IBM, Bioscan, INC., Washington). The radioactivity of the tritiated metabolites was also determined as cpm counts by the scanner. The positions of the non-radiolabelled steroids on the developed TLC sheets were identified using fluorescence or iodine vapours. Illuminating the sheets with 254 nm ultraviolet light made the Δ^4 -3-ketosteroids visible and iodine vapour was used to visualise the 5 α -reduced forms i.e., 5 α -A and DHT that stained as dark spots. The R_f value of each steroid was recorded.

3.2.8.2. Oestradiol radioimmunoassay of androstenedione metabolites

Oestradiol was measured in non-extracted culture medium as described (Hillier & De Zwart, 1981). The inter-assay coefficients of variation for the "low" (50 pg/100 μ l) and "high" (100 pg/100 μ l) quality control solutions were 17.0% and 18.2%, respectively. The intra-assay coefficient of variation was 8% for both solutions.

3.2.9. Data Analysis

The results of the oestradiol RIAs were calculated using AssayZap Universal Assay Calculator (Biosoft Ltd., Cambridge, UK). The steroid levels of the experimental treatments were analysed by one way ANOVA and student's t-test after statistical computation with Microsoft Excel computer software. Graphical presentation was done using Cricket Graph computer software (Cricket Software, Malvern, PA, USA).

3.3. RESULTS

3.3.1. Evidence for Androgen Aromatisation by Granulosa Cells

To study aromatisation of androstenedione by granulosa cells, metabolism of [3 H]androstenedione and unlabelled androstenedione were studied by radiochromatography and specific RIA, respectively.

3.3.1.1. Radiochromatography

I. Validation of TLC system

Alumina pre-coated sheets were used initially for the separation of androstenedione metabolites by TLC because they achieved distinct separation of

androstenedione from 5 α -A, androsterone, and DHT, the main 5 α -reduced metabolites of androstenedione as shown by the developments of authentic steroid solutions by TLC (Fig.3.4; Table 3.1). Distinct separation of oestradiol was also achieved, but DHT and androsterone were not separated in this system.

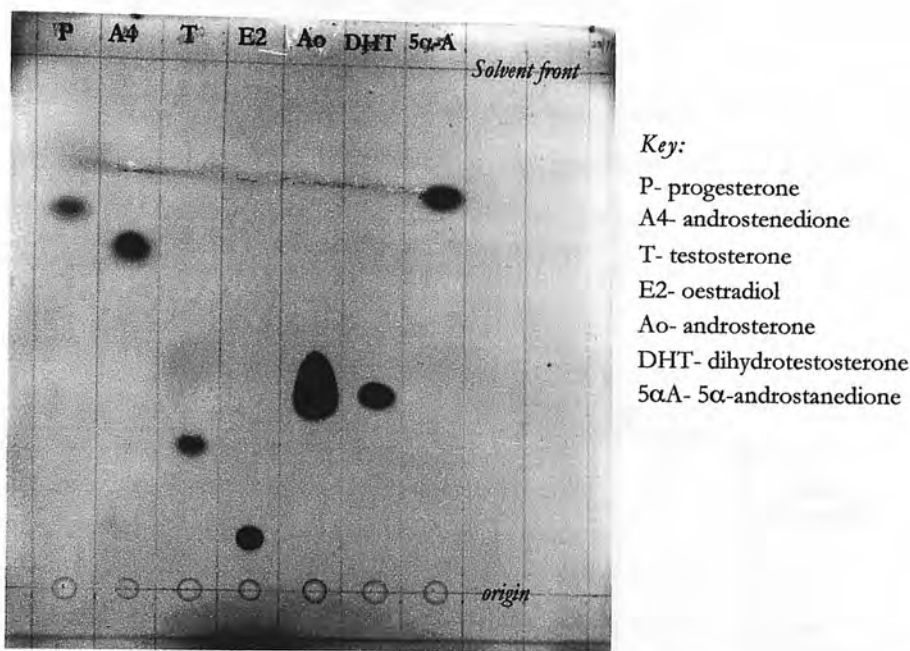


FIGURE 3.4. TLC separation of ovarian steroids on alumina pre-coated sheets. Solutions of authentic non-radiolabelled steroids were separated by TLC on alumina pre-coated sheets by development in a solvent system of cyclohexane:toluene:ethanol (100:95:5, v/v/v). Repeated separations gave similar results.

Steroid	Rf value
Progesterone	0.77
Androstenedione	0.72
Testosterone	0.32
Oestradiol	0.08
Androsterone	0.41
Dihydrotestosterone	0.42
5 α -androstanedione	0.81

TABLE 3.1. Steroid Rf values after TLC on alumina pre-coated sheets. The mobility of each steroid relative to the solvent front (Rf value) was computed after two developments on alumina sheets in the solvent system, cyclohexane:toluene:ethanol (100:95:5, v/v/v). Distance from the origin to the solvent front = 16.5 cm.

II. Effect of in vivo gonadotrophin treatment on aromatisation of androstenedione

Metabolism of [^3H]androstenedione occurred in immature and eCG-stimulated granulosa cells, as shown in Fig. 3.5. Testosterone was produced as the major steroid metabolite indicating a predominance of 17β -hydroxysteroid dehydrogenase type 1 (17β -HSD1) activity in rat granulosa cells. Oestradiol biosynthesis occurred as one of the minor metabolic processes, thereby indicating aromatase action. The oestrogens peaks were reduced ~ 30 -40% by Arimidex (Fig. 3.5 F and H). The other minor metabolites indicated as *peak 1* and *peak 2* could not be identified. It is worth noting that 5α -androstenedione was not synthesised by the granulosa cells as a product of androstenedione metabolism. Therefore, inhibition of oestrogen biosynthesis did not potentiate the alternative 5α -reductive pathway (Fig. 3.6. F and H).

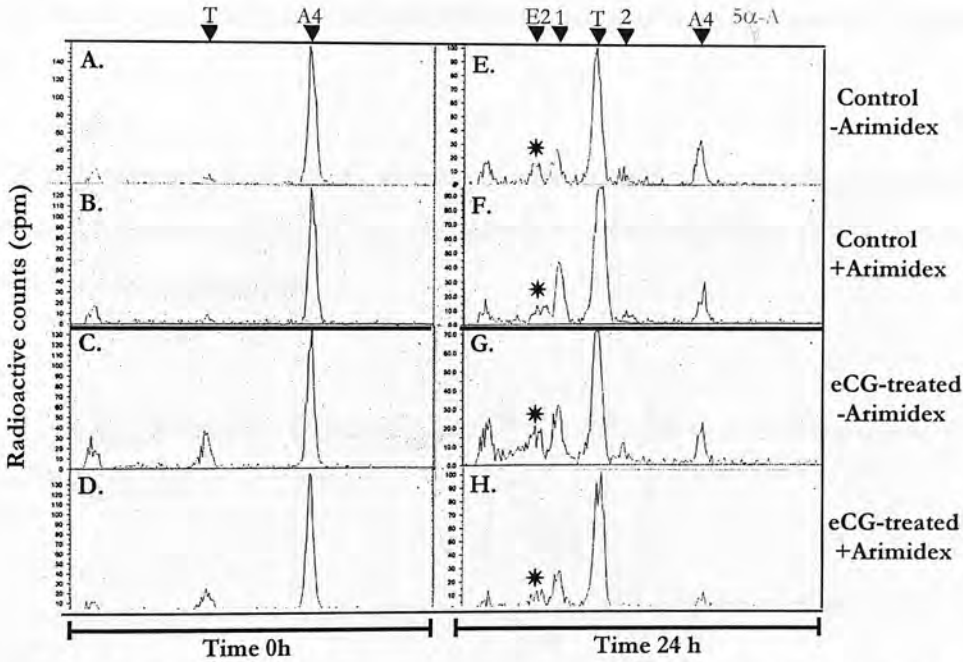


FIGURE 3.5 Granulosa cell production of steroid metabolites from $1.8\mu\text{M}$ androstenedione. Steroids were extracted from 0.5 ml granulosa cell culture medium obtained from pooled 24 h incubations of 6 replicate cultures set up with [^3H]androstenedione (A4, 1×10^5 dpm; specific activity 9.2×10^{-7} $\mu\text{Ci}/\text{mmol}$) and $1.8 \mu\text{M}$ non-radiolabelled androstenedione. Separation of the steroids by TLC on alumina pre-coated sheets showed that metabolism of androstenedione occurred in rat granulosa cell cultures soon after addition of substrate (Time 0h cultures, A-D) and after incubation for 24 h (E-H). The cell cultures contained granulosa cells from unstimulated, control ovaries in the absence (A & E) and presence (B and F) of Arimidex, and granulosa cells from eCG-stimulated ovaries in the absence (C & G) and presence (D & H) of Arimidex. The identified steroid metabolites were testosterone (T) and oestradiol (E2). (*) represents the oestradiol peak. Peaks 1 and 2 are unidentified steroid metabolites. 5α -androstenedione (5α -A) steroid metabolite was absent from the expected position. Replicate experiments ($n=3$) gave similar results.

Oestradiol, as a percentage of total androstenedione metabolites increased ~10% when the androstenedione substrate concentration was reduced to the nanomolar range (18 nM), which has been indicated to maximise aromatase activity (Brandt *et al.*, 1990) (Fig. 3.6). At this concentration, oestradiol production accounted for ~20% of the steroids synthesised from androstenedione, compared with 10% at the higher, albeit physiological, 1.8 μ M androstenedione concentration. Consistent with the metabolism of the higher concentration of androstenedione substrate, enzymic conversion of androstenedione was greater in eCG-stimulated granulosa cells (Fig. 3.6C and D) than the controls (Fig. 3.6A and B) as shown by further reduction (~50%) of the androstenedione peak in the eCG-treated panels. Arimidex reduced oestradiol synthesis (Fig. 3.6B and D). However, testosterone was not singly dominant as the main steroidal product at this substrate concentration. Oestradiol and *peak 1* were major metabolites of both immature and eCG-stimulated granulosa cells. While *peak 2* was also a major metabolite in immature granulosa cells, little was produced in eCG-stimulated granulosa cells.

Thus, oestradiol biosynthesis, as result of androstenedione metabolism by granulosa cells was demonstrated *in vitro*, but conversion of androstenedione to 5 α -A was not evident in these experiments.

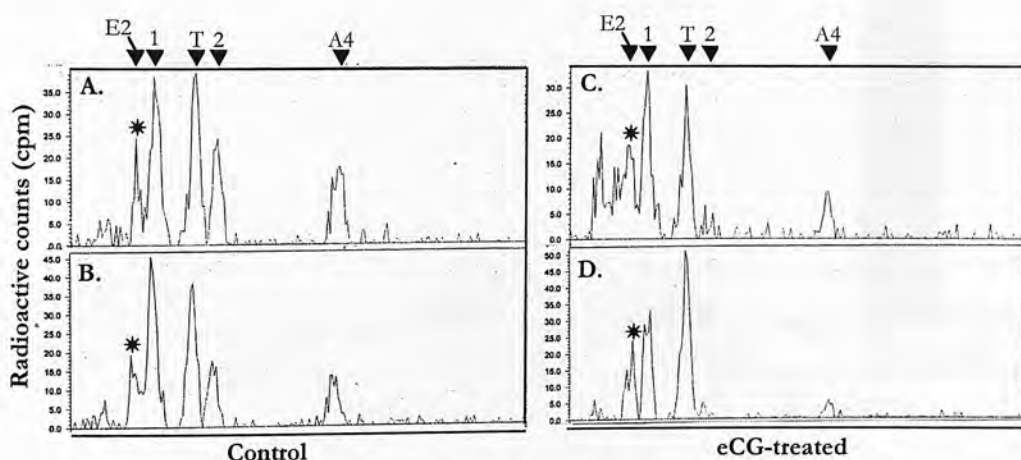


FIGURE 3.6. Granulosa cell production of steroid metabolites from a reduced (18nM) androstenedione substrate concentration. After 24 hour cell cultures, steroid biosyntheses in pooled culture medium of immature (control) and eCG-treated granulosa cell cultures containing [3 H]androstenedione (A4; 1×10^5 dpm; 9.2×10^{-7} μ Ci/mmol) and 18 nM non-radiolabelled androstenedione substrate were demonstrated after TLC on alumina sheets. Oestradiol (E2) biosynthesis (*) was illustrated in the absence (A and C) and presence (B and D) of Arimidex in the cell cultures. Testosterone (T) and the unidentified steroids, *peaks 1* and *2* were produced in the granulosa cell cultures.

3.3.1.2. Oestradiol radioimmunoassay

Oestradiol biosynthesis, as a product of non-radiolabelled androstenedione metabolism (at a substrate concentration of $1.8\ \mu\text{M}$), was significant in the 24-h incubations of granulosa cells from immature, unstimulated ovaries (controls; $P < 0.001$) and eCG-stimulated ovaries ($P < 0.001$) as shown in Fig. 3.7. However, oestradiol biosynthesis was significantly greater in granulosa cells from eCG-stimulated ovaries compared to the control ovaries whether at time 0 h ($P < 0.0001$) or 24 h ($P < 0.001$) in the absence of Arimidex. Arimidex significantly reduced oestradiol biosynthesis in the 24-h cultures of granulosa cells from both control ($P < 0.001$) and eCG-stimulated ($P < 0.001$) ovaries.

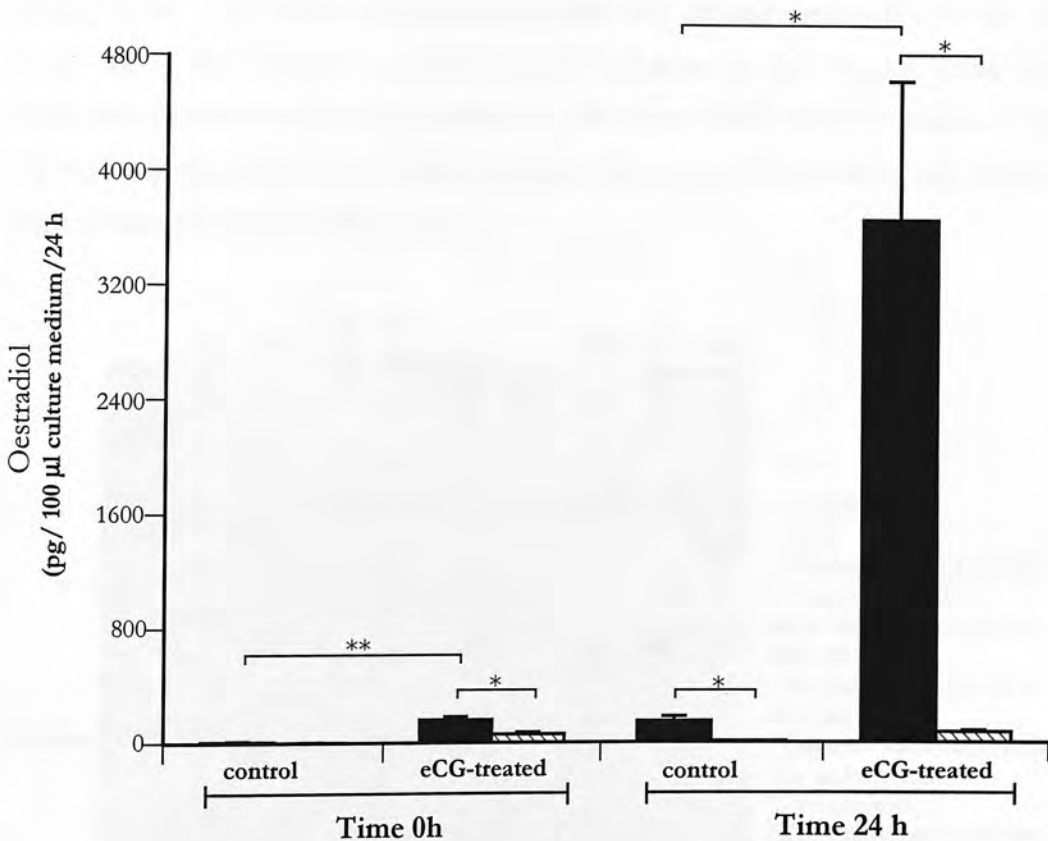


FIGURE 3.7. Oestrogen biosynthesis by granulosa cells. Oestradiol, produced as a product of androstenedione ($1.8\ \mu\text{M}$) metabolism in non-incubated (time 0h) and 24-h incubations (24 h) of granulosa cells, was measured in duplicate by specific RIA on pooled culture medium of immature, unstimulated (control) and eCG-stimulated granulosa cell cultures containing (hatched bars) and lacking (solid bars) Arimidex. Each bar represents mean oestradiol production in 3 replicate experiments. Asterisks denote significant differences between the group means indicated (* $P < 0.001$; ** $P < 0.0001$). (ANOVA: $F=82.1$, $P < 0.0001$).

3.3.2. Evidence for Androgen 5 α -Reduction by Granulosa Cells

To study 5 α -reduction of androgens by ovarian cells, metabolism of [3 H]androstenedione was studied by radiochromatography using a modified TLC system, designed to resolve multiple 5 α -reduced androgen metabolites.

3.3.2.1. Validity of TLC system

Silica pre-coated sheets developed with dichloromethane:ethyl acetate:95% ethanol (100:95:5, v/v/ v) were used for the subsequent separations of the steroid metabolites because in studies of aromatisation, it was noted that alumina pre-coated sheets were problematic in the complete separation of [3 H]androstenedione metabolites (Figs. 3.5 and 3.6). 5 α -A was separated distinctly from androstenedione and its hydroxylated form, 5-androstene-3 β ,17 β -diol (A-diol), and other 5 α -reduced androgens, androsterone, DHT, 5 α -androstane-3 α ,17 β -diol and 5 α -androstane-3 β ,17 β -diol, as shown by the developments of authentic steroid solutions by TLC (Fig.3.8; Table 3.2). Separation of testosterone was also achieved. However, DHT and androsterone were incompletely separated, and 5 α -androstane-3 α ,17 β -diol and 5 α -androstane-3 β ,17 β -diol were indistinguishable by this system.

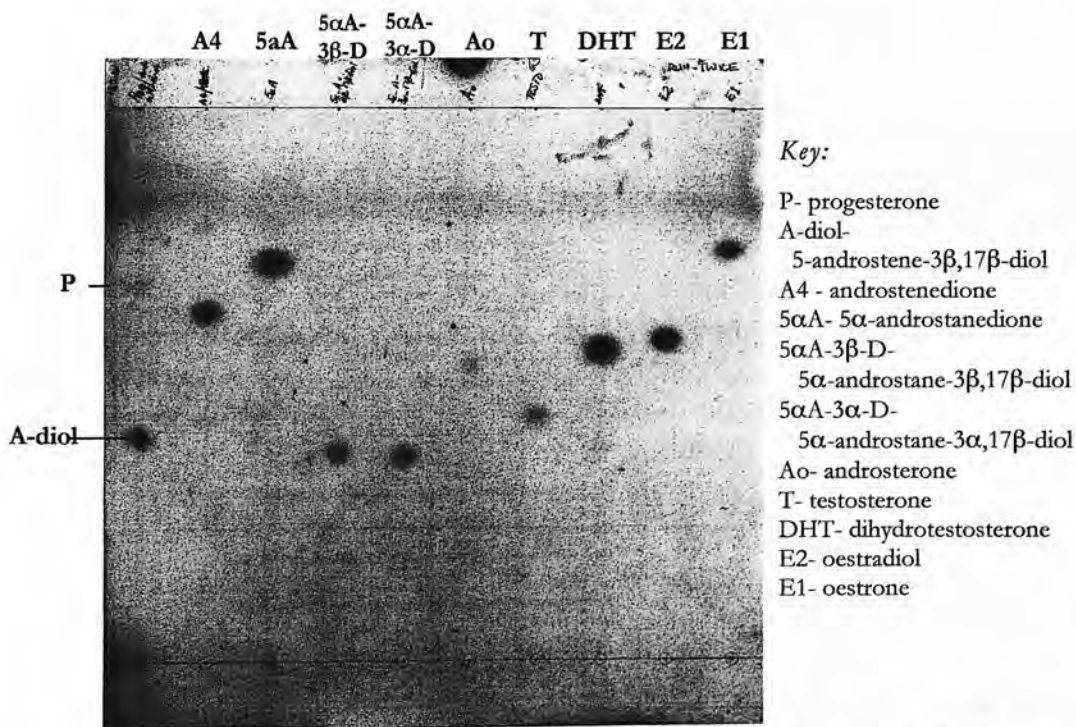


FIGURE 3.8. TLC separation of ovarian steroids on silica pre-coated sheets. Solutions of authentic non-radiolabelled steroids were separated by TLC on silica pre-coated sheets by development in a solvent system of dichloromethane:ethyl acetate:95% ethanol (100:95:5, v/v/ v). Repeated separations gave similar results.

Steroid	Rf value
Progesterone	0.68
5-androstene-3 β ,17 β -diol	0.41
Androstenedione	0.63
Testosterone	0.44
Oestradiol	0.55
Androsterone	0.54
Dihydrotestosterone	0.56
5 α -androstenedione	0.72
5 α -androstane-3 β ,17 β -diol	0.38
5 α -androstane-3 α ,17 β -diol	0.37
Oestrone	0.74

TABLE 3.2. Steroid Rf values after TLC on silica pre-coated sheets. The mobility of each steroid relative to the solvent front (Rf value) was computed after two developments on silica pre-coated sheets, in the solvent system of dichloromethane:ethyl acetate:95% ethanol (100:95:5, v/v/ v). (Distance from the origin to the solvent front = 16.5 cm).

3.3.2.2. Effect of in vivo gonadotrophin treatment on 5 α -reduction of androstenedione

Since ovarian 5 α -reductase expression was previously shown to be developmentally regulated, at least in adult rat ovary (Lephart *et al.*, 1992), granulosa cells from ovaries induced to attain follicular development by treatment with eCG (see "Experimental Procedures", 3.2.1) were used to investigate 5 α -reduction *in vitro*, comparing metabolism of androstenedione and testosterone as substrates.

5 α -A was not synthesised from either androstenedione (Fig. 3.9A) or testosterone (Fig. 3.9B). Nor was testosterone converted to DHT, whereas oestradiol synthesis clearly occurred (Fig. 3.9B). Therefore, once again, 5 α -reduction could not be demonstrated in granulosa cells. However, the formation of testosterone as the principal androstenedione metabolite in stimulated ovaries again indicated 17-oxo-reduction by 17 β -HSD1 as the predominant route of androgen metabolism by granulosa cells of preovulatory follicles (Fig. 3.9A). Although, 5 α -reduction could not be demonstrated in granulosa cell cultures, the concentration of non-radiolabelled androstenedione substrate used (1.8 μ M) was within the Km range (1 - 5 μ M) of 5 α -reductase for steroid substrates reported in other studies (Russell and Wilson, 1994).

To check the influence of substrate concentration on 5 α -R activity in granulosa cells, androstenedione at concentrations of 0.03, 0.3, 3.0, 30 or 300 μ M was incubated for 24 h with a constant number (1×10^5) of viable granulosa cells from unstimulated

control ovaries, but 5 α -A could not be identified as a steroid metabolite. In fact, the 30 and 300 μ M substrate concentrations inhibited all enzymic action of the granulosa cells (data not shown).

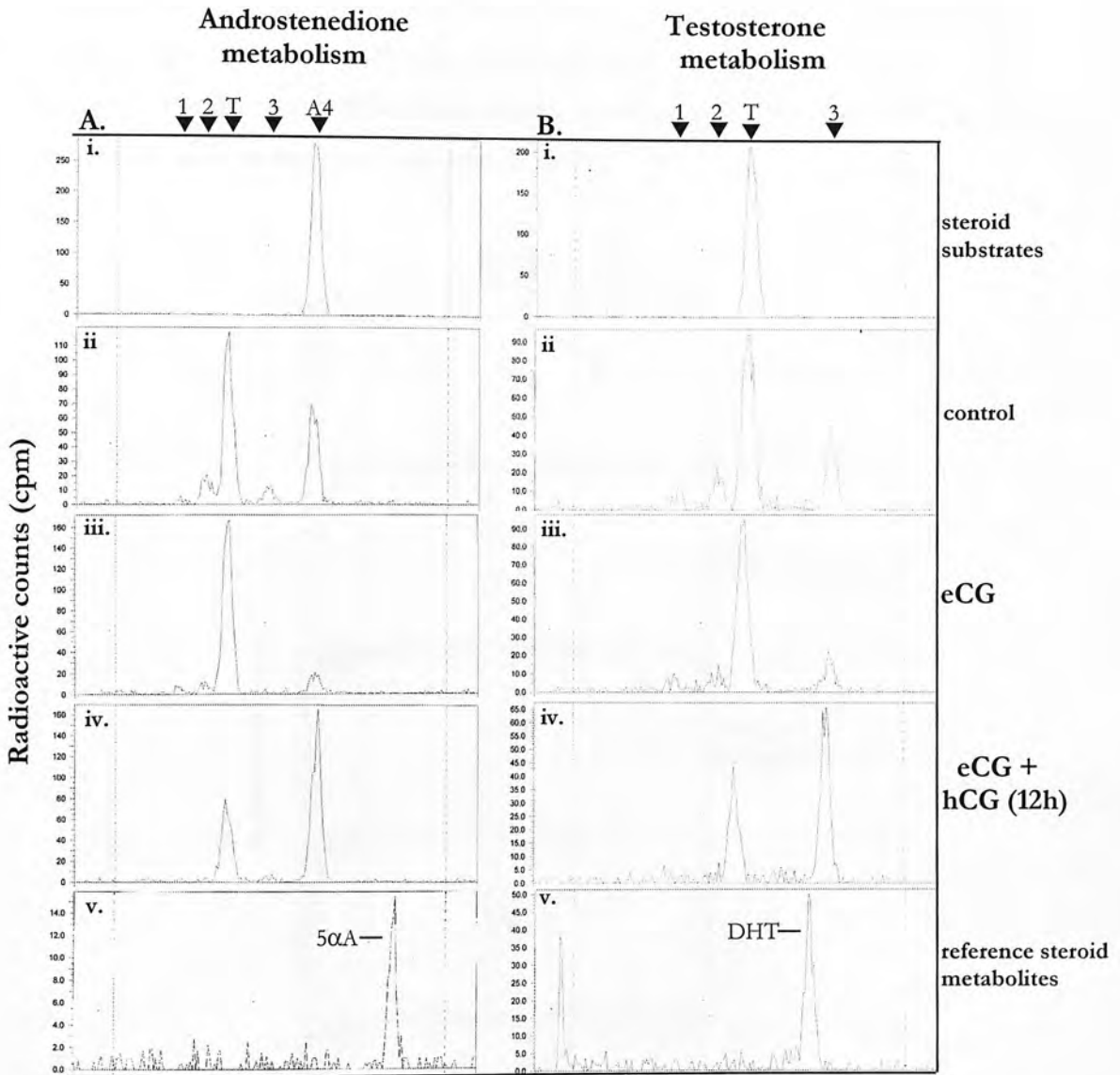


FIGURE 3.9. Androgen metabolism by granulosa cells at different stages of follicular development. Granulosa cells isolated from ovaries stimulated *in vivo* (see "Experimental Procedures, 3.2.1) were incubated for 24 h with either 1.8 μ M androstenedione (A) or 1 μ M testosterone (T) as substrate. Conversion of the steroid substrates (i) into respective 5 α -reduced steroid metabolites (v), 5 α -androstenedione (5 α A) and dihydrotestosterone (DHT) was investigated in control (ii), eCG-stimulated (48-h treatment) (iii), and eCG followed by 12-h treatment with hCG (iv). Separation of the steroids was done on silica pre-coated sheets developed with dichloromethane:ethyl acetate:95% ethanol (100:95:5, v/v/v). Peaks 1, 2 and 3 are unidentified steroids, but peak 3 is a composite peak made up of oestradiol and other unidentified steroids.

3.3.3. EVIDENCE FOR 5 α -REDUCTION IN WHOLE OVARY

Despite the inability to demonstrate 5 α -reduction in isolated granulosa cells, homogenates of whole ovary clearly possessed this enzymic activity. Androsterone, as shown by radiochromatography, was the main product of androstenedione metabolism in immature rat ovary, thereby indicating 5 α -R activity (Fig.3.10). Although, homogenates of whole ovary, unlike the granulosa cells, metabolised androstenedione substrate concentrations of 30 μ M, the 5 α -reductive conversion of androstenedione was greater at 3 μ M substrate concentration, which approximates the K_m of the rat enzyme for androstenedione (Andersson & Russell, 1990).

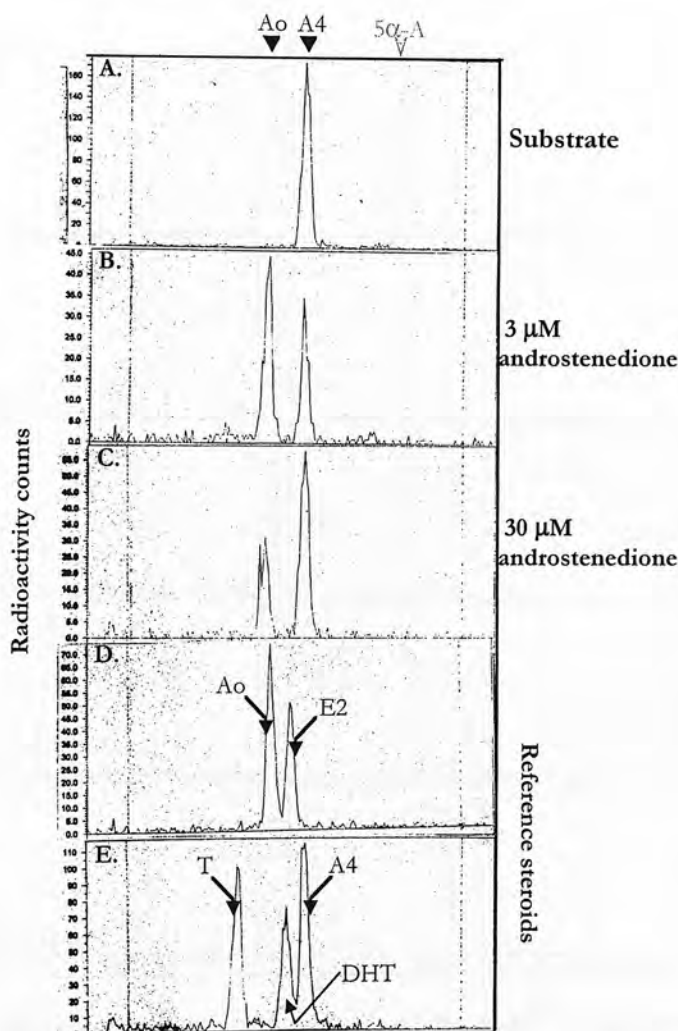


FIGURE 3.10. Identification of steroid products of androstenedione metabolism by whole ovarian homogenates. Homogenates representing 2 mg immature ovary were incubated (see Experimental Procedures, 3.2.6) with [3 H]androstenedione (1×10^5 dpm) and either (B) 3.0 μ M or (C) 30 μ M non-radiolabelled androstenedione substrate. Identification of androsterone (Ao), the main metabolic product in immature rat ovary was achieved by developing extracts of the homogenates alongside the authentic tritiated steroids, oestradiol (E2), testosterone (T), dihydrotestosterone (DHT) and androstenedione (A4) on silica pre-coated TLC sheets. 5 α -androstenedione (5 α -A) was among the expected metabolites.

By adapting a method designed to investigate 5 α -R1 activity *in vitro* (Terakawa *et al.*, 1978), 5 α -R1 activity was clearly demonstrated in whole ovary homogenates incubated with [3 H]androstenedione as substrate (Fig. 3.11A). The main component in composite

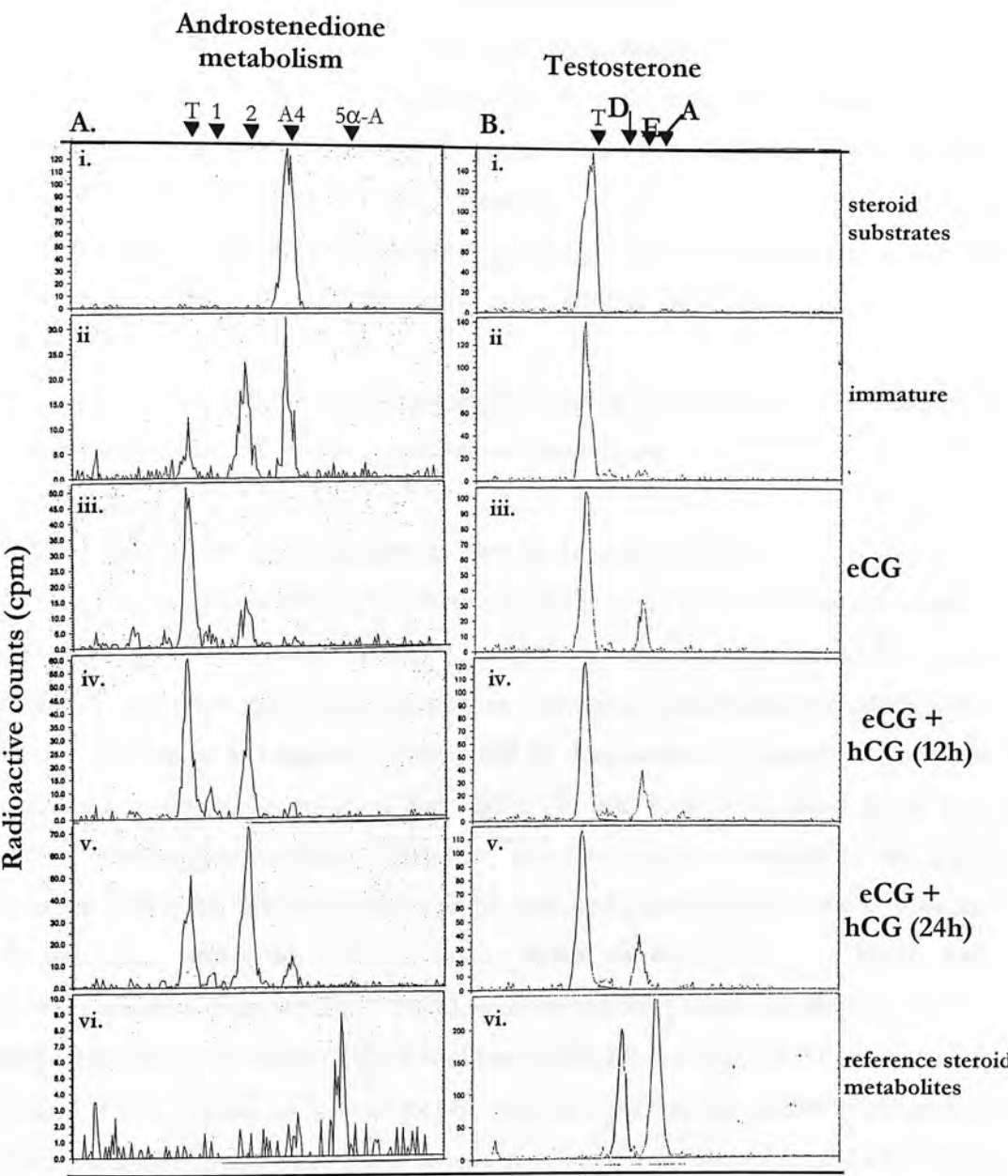


FIGURE 3.11. 5 α -reductase activity assay in whole ovary at different stages of development. Adapting the method used by Terakawa *et al.* (1978), homogenates of whole ovaries collected from rats stimulated as indicated ovaries were incubated for 30 minutes with $\sim 1 \times 10^5$ dpm counts of [3 H]androstenedione (A) or [3 H]testosterone (B) as substrate. Conversion of steroid substrates (i) into 5 α -reduced steroid metabolites (vi) was investigated in control (ii), eCG-stimulated (48-hour treatment) (iii), eCG followed by 12-hour treatment of hCG (iv) and eCG followed by 24-hour treatment of hCG (v). Separation of the steroid metabolites was done on silica pre-coated sheets. Peak 1 is an unidentified steroid. Peak 2 is a composite peak made up of oestradiol, androsterone and other unidentified steroids.

peak 2 of ovaries from immature, untreated rats (Fig. 3.11Aii) was androsterone as shown in Fig. 3.10. Production of androsterone-containing peak 2 was decreased ~40% (compared to control value) by eCG (Fig. 3.11A iii), but was increased by hCG (Figs. 3.11A iv and v) indicating the inhibitory and stimulatory effects of eCG and hCG, respectively, on the action of 5 α -R1. However, consistent with metabolic studies of granulosa cells, the main enzyme activity in rat ovary stimulated by gonadotrophins was again shown to be 17 β -HSD1, as shown by the production of testosterone as the principal metabolite of eCG- and hCG-treated ovaries (Fig. 3.11 iii and iv). Similarly, too, 5 α -A was not shown as a major metabolite of androstenedione (Fig. 3.11A) or testosterone (Fig. 3.11B), even though oestradiol was clearly synthesised in stimulated ovaries (Fig. 3.11B iii-v). Nor was testosterone converted to DHT.

Therefore, although whole ovaries of immature rats had 5 α -reductive activity, it was essentially absent from the granulosa cell compartment.

3.3.4. Evidence for 5 α -Reduction in Thecal/Interstitial Cells

The inability to identify 5 α -reductase activity in isolated granulosa cells when it was clearly present in whole ovarian homogenates suggested that the activity might reside in another ovarian cellular component. However, thecal/interstitial cells whether freshly isolated or LH-treated *in vitro*, failed to demonstrate 5 α -reductive activity in cultures containing physiological and higher (10-fold K_m) concentrations of non-radiolabelled androstenedione. Therefore, as a final resort, a comparison was made between androgens (testosterone and androstenedione), and progesterone as potential 5 α -reductase substrates. Viable thecal/interstitial cells were incubated with concentrations of progesterone (0.5 μ M), androstenedione (2.8 μ M) or testosterone (2.5 μ M) which are approximate to the K_m of the rat enzyme for each steroid (Andersson & Russell, 1990). However, steroid metabolism did not occur as shown by absence of other metabolite peaks after 2 h-incubations of steroid substrates (Fig. 3.12). Non-radiolabelled steroid 20 α -dihydroprogesterone (20 α -DHP) was also used as a steroid substrate for 5 α -reductase activity in thecal/interstitial cells, but no metabolism occurred (results not shown). The absence of authentic radiolabelled 20 α -DHP made it difficult to locate the steroid on thin-layer chromatograms.

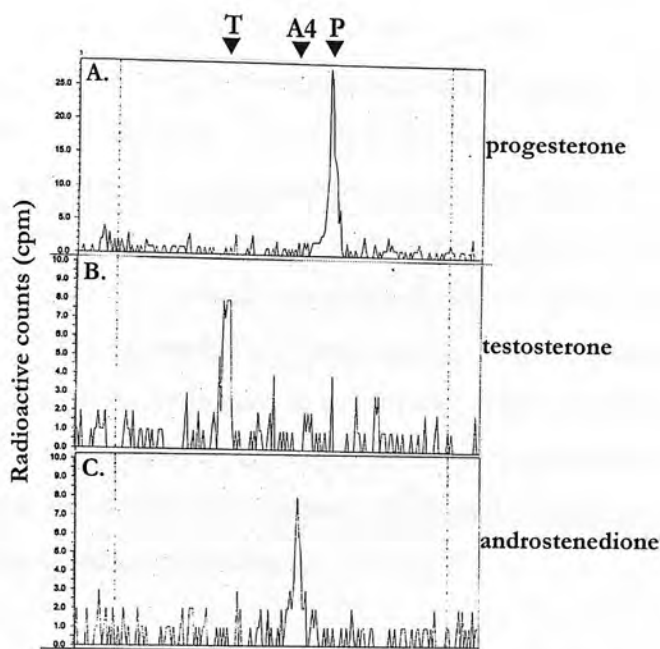


FIGURE 3.12. 5 α -reductase assay of thecal/interstitial cells using different steroid substrates at physiological Km. As described (Experimental Procedures, 3.2.6), 5 α -reductase activity was studied in viable thecal/interstitial cells (5×10^5), but metabolism of the substrates did not occur. Enzymic reactions (1 mM NADPH, 2 hours at 37°C) in ruptured cells prepared in assay buffer contained [^3H]steroid and non-radiolabelled steroid substrates; A, 0.5 μM progesterone (P); B, 1 μM testosterone (T); C, 3 μM androstenedione (A4).

3.4. DISCUSSION

This study shows that short-term granulosa cell cultures are suitable systems in which to study androgen metabolism by ovarian enzymes, with a 24-h incubation being adequate for major metabolites to accumulate. Under these conditions, granulosa cells metabolised androstenedione, an aromatisable androgen, into several steroid metabolites which included testosterone, 17 β -oestradiol and androsterone. Metabolism to differing degrees occurred in cultures of both immature and hormonally-stimulated granulosa cells. However, although thecal/interstitial cells synthesise steroids *in vitro* (Payne *et al.*, 1992), the culture system used here was unable to show steroid metabolism by these cells.

It is now well established that metabolism of androgens by granulosa cells is central to the steroidogenic function of the mammalian ovary undergoing preovulatory follicular developments (Gore-Langton & Armstrong, 1988). Granulosa cells in preovulatory follicles are enzymically equipped to metabolise androgens into a variety of

steroids, the principal endocrine secretion being 17β -oestradiol (Hillier, 1985; Richards, 1994). The increased ability of eCG-treated granulosa cells to metabolise androstenedione (Figs. 3.5 and 3.6) is reminiscent of the changes that occur during preovulatory follicle development. As preovulatory ovarian follicles develop due to FSH action, they increasingly convert androgenic precursors into oestrogens (Hillier, 1985) as shown by the dramatic increase in oestradiol synthesis by the eCG-stimulated granulosa cells (Fig. 3.7). FSH induces this action during ovarian follicle development by up-regulation of the expression of steroidogenic P450 aromatase gene which synthesises oestrogens from androgens *in vivo* (Hillier, 1985; Richards, 1994). This response fulfils the requirement of increasing levels of oestrogen that drive further preovulatory follicle growth and development, preparing the uterus for pregnancy and initiating the ovulation-inducing LH surge.

The main androgens for oestrogen synthesis in follicular fluid, androstenedione and/or testosterone, are increasingly converted into oestrone and oestradiol, respectively. In man and rat, as in most species studied, androstenedione is the main substrate for aromatisation (Gore-Langton & Armstrong, 1988). In this study, the immediate and significant production ($P < 0.0001$) of oestradiol by non-incubated eCG-stimulated granulosa cells (Fig. 3.7) suggested that stimulation by eCG raised the potency of aromatase to such an extent that it readily converted androgenic substrates into oestradiol. Therefore, during follicle development, FSH-induced aromatase action readily converts androstenedione into intermediate steroids, oestrone or testosterone, which are sequentially metabolised into 17β -oestradiol (see Chapter 1, Fig. 1.2).

In rat ovary, the favoured androgen metabolic pathway involves the enzyme 17β -HSD which is expressed constitutively in granulosa cells (Bogovich & Richards, 1984). Steroid 17β -HSD1 belongs to a class of enzymes possessing 17β -hydroxysteroid dehydrogenase/ 17 -ketosteroid reductase (17HSD/KSR) activities (Peltoketo *et al.*, 1999). The specific 17β -HSD isozyme present in ovarian follicles, 17β -HSD type 1 (17β -HSD1) (Zhang *et al.*, 1996) is the principal isozyme involved in oestradiol production both in humans and rodents (Poutanen *et al.*, 1995). Generally, 17β -HSD1 is essentially an enzyme of oestradiol synthesis and abundantly expressed in granulosa cells (Peltoketo *et al.*, 1999). In this study, its action was illustrated by the synthesis of

testosterone as the principal metabolite of androstenedione metabolism by rat ovary (Fig. 3.11), specifically the granulosa cells (Figs. 3.5, 3.6 and 3.9). Steroid 17 β -HSD1 readily converts androstenedione into testosterone prior to its aromatisation to oestradiol. The action of 17 β -HSD action is up-regulated by FSH, as indicated by the increase in the testosterone peak in eCG-stimulated ovaries (Fig. 3.11). Although the human enzyme prefers phenolic substrates over neutral ones (Peltoketo *et al.*, 1999) and hence, androstenedione metabolism transpires via oestrone, then oestradiol (Gore-Langton & Armstrong, 1988), the rodent enzyme catalyses effectively both oestrogenic and androgenic substrates (Peltoketo *et al.*, 1999). Thus, androstenedione metabolism in rodents occurs through formation of testosterone which is converted into oestradiol. However, in cultured cells that reflect *in vivo* conditions, 17 β -HSDs are mainly 17HSDs or 17KSRs (Peltoketo *et al.*, 1999). This explains why the reductive reaction converting testosterone substrate into androstenedione was not found in granulosa cell cultures even though similar cultures converted androstenedione substrate into testosterone by the oxidative reaction of 17 β -HSD1. By comparison, the favoured pathway of oestradiol synthesis in humans is via aromatisation of androstenedione into oestrone, which is then converted into oestradiol by 17 β -HSD (Peltoketo *et al.*, 1999). Regardless of the steroidogenic pathway involved, the dominant steroid synthesised in preovulatory ovaries is oestradiol. Thus, the findings in the granulosa cell cultures were concordant with the current concepts of androgen metabolism in the ovary.

In subsequent studies described in this thesis, theca and interstitial cells are evidently shown to be the specific ovarian cell types expressing 5 α -R1 (Chapters 3 and 4). This would explain why 5 α -reduction of androstenedione into 5 α -A could not be demonstrated as the immediate product of androstenedione metabolism by granulosa cell cultures. Although whole ovary homogenates were shown to synthesise androsterone (Fig.3.10), thereby indicating 5 α -R activity and sequential 3 α -HSD metabolism of 5 α -A), the high K_m of 5 α -R for androstenedione (2.8 μ M; (Andersson & Russell, 1990) suggests minimal direct conversion of androstenedione into 5 α -A via the 5 α -reductive pathway. The abundant expression of 17 β -HSD1 in ovarian follicles (Zhang *et al.*, 1996) that is induced by FSH (Poutanen *et al.*, 1995), readily channels the metabolism of androstenedione through testosterone as a steroid intermediate. It is, therefore, more likely that sequential metabolism of androstenedione via testosterone

which is 5 α -reduced to DHT before conversion to 5 α -androstenediol-3 α ,17 β -diol was the favoured pathway leading to androsterone production (see Chapter 1, Fig. 1.4). Physiologically, however, the 5 α -reductive pathway possibly favours conversion of progestins as shown by the synthesis of detectable amounts of 5 α -reduced progestins from 20 α -dihydroprogesterone and progesterone *in vitro* (Lerner & Eckstein, 1976; Payne *et al.*, 1992). The low K_m of 5 α -R1 for progesterone (0.5 μ M) (Normington & Russell, 1992) favours 5 α -reduction of progesterone over androgen reduction by 5 α -R1. The detection of high levels of 5 α -reduced progestins *in vivo* (Inaba *et al.*, 1978) and in follicular fluid (McNatty *et al.*, 1979; McNatty *et al.*, 1981) seems to support this. However, it is likely that in conditions where follicular levels of androstenedione are greatly elevated such as in PCOS, some of the androstenedione escapes metabolism via the principal Δ^4 or Δ^5 pathways and is metabolised via the alternative 5 α -reductive pathway, as suggested by Agarwal *et al.*, 1996.

The synthesis of androsterone as the main product of androstenedione metabolism in immature ovaries (Fig. 3.10) correlates with the high activity of the enzyme in immature ovaries (Fukuda *et al.*, 1979; Aono *et al.*, 1981). The immediate product, 5 α -A was not detected presumably because it was converted to androsterone due to the action of 3 α -HSD. Similar effects of 3 α -HSD action have been reported by (Payne *et al.*, 1992), and therefore, the absence of 5 α -A in cultures of whole ovary homogenates did not signify absence of 5 α -R activity, but swift conversion of the immediate product, 5 α -A into androsterone. Mammalian 3 α -HSDs work in concert with 5 α - and 5 β -reductases to produce 5 α ,3 α - and 5 β ,3 α -tetrahydrosteroids (Penning, 1997). An example is the conversion of 5 α -DHT by 3 α -HSD into 3 α -androstenediol, which has a much lower affinity for the androgen receptor. Therefore, the 5 α -reductive metabolism of androstenedione followed by 3 α -HSD action results in the formation of steroids with less androgenic ability. This is important physiologically in immature rats where precocious synthesis of oestrogens is inhibited by formation of androsterone. Being non-aromatisable, androsterone can not be converted to oestrogens. It is also an aromatase inhibitor (Adashi, 1994) and its property as a 5 α ,3 α -tetrahydrosteroid reduces its affinity for the androgen receptor. Therefore, it not only constrains the formation of oestrogen, but also has weak potential as a paracrine androgen such that the atretic androgenic effects are somewhat inhibited possibly allowing immature

follicles to remain undeveloped but viable and potentially able to be recruited as preovulatory follicles after puberty.

The change in the patterns of androgen metabolism and the ensuing steroid biosyntheses reflect the changing steroidal requirements of developing follicles. Although androsterone was not resolved in Fig. 3.11, prior identification had shown it to be the major metabolite of androstenedione in immature ovaries (Fig. 3.10). Hence, studies of androstenedione metabolism in homogenates of whole ovaries at different stages of follicular development (Fig. 3.11), indicate that androsterone was a major component in peak 2 of immature, unstimulated ovaries (Fig. 3.11ii). This is in support of earlier studies where the main androgenic metabolites of ovaries from immature female rats at 20 and 28 days age were androsterone and 5α -androstane- $3\alpha,17\beta$ -diol, (Karakawa *et al.*, 1976). As immature follicles develop, androsterone biosynthesis decreased transiently while testosterone synthesis increased due to FSH-mediated actions (Fig. 3.11A i and ii). This is crucial in preovulatory steroidogenesis because developing follicles critically require oestrogens, specifically oestradiol, to complete follicular maturation. Hence, synthesis of aromatisable steroid intermediates such as testosterone that can be converted into oestrogens overrides the synthesis of the non-aromatisable 5α -reduced androgens. As follicles approach ovulation, androsterone synthesis rose again suggesting a role of 5α -reductive metabolism during the peri-ovulatory period. The dominant hormone at this stage of the cycle increasingly becomes progesterone, but it is not yet clear why the ovary requires 5α -reduced steroids, presumably 5α -reduced progestins at this stage.

The present focus on androstenedione metabolism illustrates the difficulty of targeting specific steroidogenic pathways for study. Most steroid-metabolising enzymes act on more than one substrate, and thus the flow of steroids through any one pathway is highly dependent on the relative efficiency (substrate specificity) of the enzymes for the steroids. In addition, enzyme activity in experimental culture conditions is determined greatly by the substrate concentration. Suppression of granulosa cell metabolism of androstenedione by concentrations that were 10-fold the physiological K_m or higher, showed the importance of substrate concentration in experimental procedures investigating enzyme activity. Androstenedione concentrations within the

nM range were optimal for most ovarian enzymes as shown by the increased number and levels of steroid metabolites synthesised from androstenedione when the substrate concentration was 18 nM (Fig.3.6). High levels of substrate become inhibitory to enzyme action, and this is the reason why the levels of substrates in physiological processes such as steroid metabolism are tightly regulated within narrow physiological ranges, which ensure enzymic action. Thus, androgens, although being the steroid precursors of ovarian oestrogens, also have a kinetic role in regulating the enzymatic processes within the ovary. This dual role, which embodies paracrine action, can only operate normally if androgen biosynthesis is properly balanced with metabolism such that no accumulation of androgens occurs to offset the finely tuned intraovarian processes, which ensure specific steroid formation at different stages. A classic example of the effects of altered ovarian steroidogenesis, particularly androgen metabolism, is the endocrine disorder, PCOS which is studied in chapter 6.

In summary, metabolism of androstenedione in rat ovary occurs in granulosa cells where aromatisation of androgens into oestradiol occurs among the synthesis of other steroids. The patterns of the steroids synthesised from androgenic precursors change as preovulatory development progresses. Synthesis of androsterone, a 5 α -reduced androgen in immature ovaries changes to synthesis of testosterone, an aromatisable steroid from which oestradiol is synthesised. While oestradiol completes follicular maturation, the rise in 5 α -reduced steroids during the peri-ovulatory window suggests a role of these steroids at this stage in the oestrous cycle. This opens up further scopes for research in the role of 5 α -reduced steroids during and around ovulation.

Chapter 4. Gonadotrophic Regulation of Rat 5 α -Reductase Type 1 mRNA Expression during the Preovulatory Period.

4.1. INTRODUCTION

5 α -Reductase is a component of the intra-ovarian steroidogenic pathways which determine steroid synthesis and secretion (Clarke, 1986; Gore-Langton & Armstrong, 1988). The enzyme also exists as the 5 β -isomer, 5 β -R (Lisboa & Holtermann, 1976; Charbonneau & Luu-The, 1999;2001). Both isoforms are expressed in several organs where they inactivate $\Delta^{4,5}$ -3-oxo-steroids in preparation for excretion (Russell & Wilson, 1994; Charbonneau & Luu-The, 2001). Whereas the detection of 5 β -reductase activity illustrated the expression of the isomer in the foetus (Stern *et al.*, 1975), males (Ingelman-Sundberg, 1976), non-reproductive tissues (Collins & Cameron, 1975; Stenberg, 1976; Hutchison & Steimer, 1981), and in adult chicken ovary (Gomez *et al.*, 1998), no evidence suggests a role for the 5 β -isoform in steroidogenesis of the adult mammalian ovary.

5 α -Reductase is encoded by two genes encoding two different isozymes designated as type 1 (5 α -R1) and type 2 (5 α -R2) which have different biochemical properties and tissue distributions (Russell & Wilson, 1994). The synthesis and detection of 5 α -reduced steroids in follicular fluid suggest an active intra-follicular role of the 5 α -R enzymes in metabolising ovarian steroids, and hence modulating ovarian function at the paracrine level (Lerner & Eckstein, 1976; Inaba *et al.*, 1978; Aono *et al.*, 1981; Haning *et al.*, 1996). Whereas the activity of the enzyme was found to be higher in immature compared to adult ovaries (Karakawa *et al.*, 1976), studies on the expression of the 5 α -R gene in immature ovary are still inconclusive. Reports of the expression of the 5 α -R mRNA gene illustrate its regulation in adult ovary by gonadotrophic stimuli (Lephart *et al.*, 1992). While studies by Aono *et al.*, 1981 suggested a similar gonadotrophic role in immature ovaries, the expression of the 5 α -R1 mRNA, the isozyme expressed in the ovary, has not yet been demonstrated.

During androgen metabolism, 5 α -reduction is an alternative pathway to aromatisation, which is critical for maturation of ovarian follicles beyond early antral stages (McGhee and Hsueh, 2000). It is therefore, one of the intraovarian mechanisms

that control/impinge on the aromatisation step, and hence critically important in preovulatory development. As part of a programme investigating 5 α -reduced androgens in the ovary, identification of the 5 α -R isozymes present and their gene expression in rat ovary provides insights to understanding the role of 5 α -R in ovarian physiology, and its regulation of oestrogen production which is pivotal to follicle development, and hence fertility.

Using ovarian tissues from immature and hormonally-stimulated rats, this study investigated the expression of 5 α -R1 and 5 β -R genes in rat ovary by reverse transcription polymerase chain reaction (RT-PCR) and ribonuclease protection assay (RPA). The expression of 5 α -R1 mRNA as a "competitor" with aromatase mRNA for androgen metabolism was investigated during preovulatory development.

4.2. EXPERIMENTAL PROCEDURES

4.2.1. Rat Tissues.

Ovarian tissue, used in reverse transcription polymerase chain reaction (RT-PCR) and ribonuclease protection assays (RPA) was obtained from intact, immature female rats injected once subcutaneously (s.c) with 10 IU eCG followed 48h later by a single injection of 10 IU hCG (s.c) and removal of ovaries after 0, 3, 6, 9, 12, 24 and 120h. Diethylstilboestrol (DES)-treated animals were injected once (s.c) with 1 mg/100 μ l DES. Control rats received no treatment. The non-ovarian tissues were collected from control rats. The tissues used in northern analyses were obtained from animals given *in vivo* treatments as described (Chapter 3, section 3.2.1). All the animals were killed by carbon dioxide asphyxiation and the tissues transported on solid CO₂ pellets prior to storage at -70°C until RNA was extracted.

4.2.2 Extraction and Quantification of Total RNA

4.2.2.1 RNA extraction using phenol-choloroform

Total RNA was extracted from rat tissues using the acid-guanidium-phenol-chloroform method of Chomczynski and Sacchi (1987). The RNA samples were dissolved in 0.5 ml DEPC-treated water and stored at -70°C.

4.2.2.2. RNA extraction using RNazol B

RNazol B (a guanidium-based reagent, TEL-TEST. Inc, Friendswood, Texas) was also used to isolate total RNA as described by the manufacturer. The isolated RNA was air-dried and dissolved in 400 μ l DEPC-treated water to which 40 μ l sodium acetate and 1.0 ml ethanol were added. The RNA samples were stored at -20°C until analysis by northern blotting.

4.2.2.3. Quantification and checking quality of RNA

Aliquots (50 μ l) of total RNA suspensions in sodium acetate/ethanol were transferred into autoclaved 0.2 ml Eppendorf tubes. After centrifugation at 21,000 g for 15 min at 4°C, the supernatant was removed, and 0.2 ml cold 85% ethanol (-20°C) was added to the tubes. Following a brief recentrifugation, purified RNA was pelleted out, then air-dried and dissolved in 10 μ l DEPC water. A gene quantifier (GeneQuant RNA/DNA calculator, Pharmacia-Biotech) was used to measure the RNA concentrations. The ratios of the optical densities of the RNA measured at 260 and 280 nm were typically greater than 1.8 and protein contamination was generally negligible, that is, less than 0.2 mg/ml.

4.2.3. Cloning of 5 α -R1 and Aromatase Complimentary DNA (cDNA)

4.2.3.1. Generation of 5 α -R1 cDNA by RT-PCR

Unless mentioned otherwise, the 5 α -R1 cDNA used was generated as explained below. Total rat liver RNA (2 μ g/5 μ l in DEPC water) isolated from immature female rats was reverse transcribed with 0.4 μ l 25 μ M 5 α -R1 antisense primer 5'-CGCTAACAGA GCACTAAAGC ACAA-3', representing amino acids 767-790 of rat 5 α -R1 gene (Anderson et al., 1989) for 1 h at 37°C. The total reaction volume (20 μ l) contained 2 μ g total RNA, 5 α -R1 AS primer (0.5 μ M-final concentration), 2 μ l 10X RT buffer, 0.4 μ l 200 μ M deoxyribose nucleotides (dNTPS), 1 μ l rRNasin (40 u/ μ l), 1 μ l MMLV reverse transcriptase (200 u/ μ l) and 10.2 μ l distilled water. The cDNA, containing a *Pst*I cleavage site at the 5'-end, was amplified with a pair of 5 α -R1 primers, 5'-CACCTCCTG GTCACCTTTG TCTT-3' (sense; amino acids 330-353 of 5 α -R1 gene) and antisense (see above) by PCR using Pfu-DNA polymerase as follows. An initial 60-s denaturing step at 94°C was followed by 29 cycles, each consisting of a 45-s

denaturing reaction at 94°C, a 45-s annealing reaction at 55°C and a 2-min extension at 72°C. The final PCR step was a 10-min extension at 72°C. Genomic DNA contamination was analysed by amplifying 1 µg RNA without RT. To verify the 5α-R1 PCR product, 10 µl of the PCR reaction were size-fractionated and visualised by electrophoresis on 2% agarose gels stained with ethidium bromide (2 µg/ml). The 5α-R1 cDNA was approximate in size to the 456 bp insert DNA fragment.

The remaining PCR reaction was size-fractionated similarly and the 5α-R1 DNA band was excised from the gel, and transferred into a 1.5 ml Eppendorf tube, which was heated at 70°C for 10 minutes. The melted agarose was processed as described (Wizard PCR Preps DNA Purification System, Promega Cat. No. A7170). Extracted DNA was eluted with 50 µl TE buffer.

4.2.3.2. Generation of aromatase cDNA by RT-PCR

Aromatase cDNA, corresponding to positions 1411 -1715 of rat aromatase gene (Hickey et al., 1990), was generated by RT-PCR as described above with the following exceptions. The primers used were 5'-ACTGGAAGAC TGTATGGATT-3' (sense) and 5'-CCAAGTCCAC GACAGGCTGA TA-3' (antisense), and the cDNA was reverse transcribed using total RNA isolated from PMSG-treated immature rat ovaries. The PCR reaction was as described above except for the 45-s annealing reaction which was performed at 60°C. The aromatase cDNA was approximate in size to the 305 bp insert DNA fragment.

4.2.3.3. Preparation of plasmid DNA expressing 5α-R1 or aromatase

The 5α-R1 and aromatase cDNAs were sub-cloned into pBluescript II SK(+) vectors, a 2961 bp bacteriophage, and transformed overnight into Epicurian coli Xli-Blue MRF' electroporation cells as described (PCR-Script™ Amp Electroporation - competent cell Cloning Kit, Stratagene Cat. No. 211186). The transformed cells were plated on agar plates supplemented with ampicillin and coated with 2% X-gal and 10 mM IPTG. Single white colonies were used to inoculate starter cultures of 5 ml LB medium containing ampicillin. Using the protocol as described (Plasmid Maxi Kit, Qiagen Cat. No. 12162), the starter cultures were used to innoculate 500 ml ampicillin-

containing LB broth from which plasmid DNA was extracted as per protocol. Plasmid DNA was dissolved in 1 ml TE buffer.

4.2.3.4. Purification of plasmid DNA using phenol-chloroform

Plasmid DNA (500 μ l) was mixed with an equal volume of phenol:chloroform:IAA (25:24:1) in 1.5 ml Eppendorf tubes which were centrifuged at 15,800 g for 5 minutes at 4°C. The top aqueous layer (~500 μ l) was transferred to fresh tubes to which an equal volume of chloroform:isoamyl alcohol (IAA) solution (49:1) was added. The tubes were recentrifuged as above and the top aqueous layer (~500 μ l) was transferred to a fresh tube. Sodium acetate (3M, 50 μ l), pH 5.2 and 1.25 ml ethanol were added to the aqueous solution and the reaction was incubated at 4°C for 2 h. After recentrifuging, the supernatant was removed and 500 μ l ice-cold 70% ethanol was added to the pellet. Following resuspension, the tubes were recentrifuged for 2 min. The ethanol was removed, and the DNA pellets were air-dried in inverted tubes for 10 min and then dissolved in 800 μ l TE buffer. The concentration of 5 α -R1 plasmid DNA (as determined by the gene quantifier) was 906 μ g/ml with an OD_{260/280} ratio of 1.849 and a protein concentration of 0.1 mg/ml.

4.2.3.5. Checking for orientation of 5 α -R1 gene insert in plasmid DNA

I. By restriction digest of plasmid DNA

In a 20 μ l reaction volume, 1 μ g 5 α -R1 plasmid DNA was mixed with the restriction enzyme, *Pst* I (1u), 1X restriction enzyme buffer H, and the total reaction volume was made up with nuclease-free water. The reaction was incubated for 1 hour at 37°C. 6X Loading Dye (4 μ l) was added to the products of the restriction digest. Untreated plasmid DNA (1 μ l) was diluted to 10 μ l with DEPC-treated water before addition of 2 μ l 6X-Loading Dye. The reaction products and untreated plasmid DNA were analysed by agarose gel electrophoresis.

II. Direct sequencing of cloned 5 α -R1 DNA

Purified 5 α -R1 plasmid DNA (300 μ g) was incorporated into a sequencing reaction as described (ABI PRISM Big dye terminator cycle Sequencing ready Reaction Kit) for a DNA thermal cycler. The products of the sequencing reaction were

precipitated using ethanol/sodium acetate protocol as described in the protocol. Electrophoresis of the samples on the ABI PRISM analysed the sequence of the cloned 5 α -R1 plasmid DNA. The identity of the cloned sequence was confirmed by a BLAST search at the National Centre for Biotechnology Information (NCBI, Bethesda, MD, USA), and the orientation of the cloned sequence was identified using Gene Jockey II Sequence Processor computer programme (Biosoft, Cambridge, UK).

4.2.4. Preparation of RNA and DNA Hybridisation Probes

4.2.4.1. ³²P-labeled RNA probes for ribonuclease protection assay

5 α -R1 and 5 β -R RNA ³²P-labeled RNA probes were synthesised by *in vitro* transcription as described (Tetsuka & Hillier, 1996) from PCR-generated 717 bp and 285 bp cDNA fragments corresponding to rat 5 α -R1 gene (location 1 to 717; (Andersson *et al.*, 1989) and rat 5 β -R gene (location 1487 to 1771; (Onishi *et al.*, 1991)). The 5 α -R1 cDNA was provided by Prof. D.W. Russell (University of Texas, Southwestern Medical Centre, Dallas, TX). Ribosomal RNA (rRNA) probes were synthesised from cDNAs containing 80 bp fragments of a highly conserved region of human 18S rRNA gene (pT7 RNA 18S; Ambion Inc). The specific activities of these probes were 6.7 x 10⁵, 6.7 x 10⁵ and 2.2 x 10³ cpm/ μ g for 5 α -R1, 5 β -R and 18S, respectively.

4.2.4.2. ³²P-labeled RNA probes for northern analyses

I. Generation of linearised 5 α -R1 plasmid DNA template

Linearised 5 α -R1 plasmid DNA (template) was generated by cleaving the plasmid cDNA as described (section 4.2.3.5. I) with the exception that the restriction enzyme, 10X buffer and incubation period used were *EcoR* V, buffer D and 2 hours, respectively. Aromatase DNA template was generated similarly with *EcoR* I and buffer H.

II. In vitro transcription of 5 α -R1 RNA probe

5 α -R1 and aromatase ³²P-labeled RNA probes were synthesised *in vitro* from linearised DNA templates as described (MAXIscript™ *In vitro* Transcription Kit, Ambion, Cat. No. 1324) using α ³²P-(Dejager *et al.*, 2001) (800Ci/mmol, 20 mCi/ml) and

purified using MicroSpin G-50 columns (Amersham Pharmacia, Cat. No. 27-5330). The probes used for detecting 18S rRNA were ^{32}P -labeled DNA probes prepared using $\alpha^{32}\text{P}$ -[CTP] (10 mCi/ml) as described (Rediprime II random prime labelling system, Amersham Pharmacia Biotech, Cat.No. RPN 1633) from a cDNA fragment corresponding to a highly conserved region of human 18S gene. The specific activities of the 5 α -R1, aromatase and 18S probes were 1.4×10^5 , 1.5×10^2 and 2.1×10^3 cpm/ μg , respectively.

4.2.5. Ribonuclease Protection Assay (RPA)

Total RNA (20 μg) was hybridised with approximately 1×10^4 cpm 5 α -R1 or 5 β -R RNA probes and 1×10^4 cpm 18S rRNA probe for 16 h at 52 °C in 20 ml hybridisation buffer containing 80% deionised formamide, 40 mM PIPES (pH 6.7), 0.4M sodium chloride and 1 mM EDTA. After hybridisation, unprotected RNA was digested by RNase A/T1 and protected fragments of mRNA/RNA were extracted by phenol/chloroform prior to precipitation by ethanol. Protected RNAs were size-fractionated by electrophoresis on 5% acrylamide gels containing 8M urea. The gels were analysed by autoradiography.

4.2.6. Northern Analysis

Total RNA (20 μg) was size fractionated in a denaturing gel, and transferred onto Hybond-N nylon membranes as described (NorthernMax™ Northern Blotting Kit, Ambion, Cat. No. 1940). The hybridisations and the subsequent washes of the northern blots, performed at 68°C and 42°C for the RNA and DNA probes, respectively, were performed as described in the protocol.

4.2.7. Data Analysis

The radioactive signals of RNA were quantified by electronic autoradiography (Instant Imager, Packard, Downers Grove, IL, USA) followed by exposure of the membranes to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) with intensifying screens for 16-48 h at -70°C. The abundance of mRNA was normalised to the 18S rRNA signal. Results of 5 α -R1 and aromatase gene expression were expressed as percentages of the control and eCG-treated values, respectively, and analysed using one way ANOVA with paired Student's t-test.

4.3. RESULTS

4.3.1. Expression of 5 α -reductase 1 and 5 β -reductase in Rat Ovary

To study the expression of 5 α -R1 and 5 β -R mRNA in the ovary (among other rat tissues), total RNA isolated from ovaries collected from immature and hormonally-stimulated rats *in vivo*, was analysed by RT-PCR and RPA.

4.3.1.1. Expression of 5 α -reductase 1 in rat tissues

5 α -Reductase-1 mRNA was expressed abundantly in the reproductive tissues, ovary uterus and testis, and non-reproductive tissues from immature female rats, liver, intestines, kidney and lung in immature rats (Fig. 4.1). The spleen and brain expressed very low levels of the enzyme. 5 α -Reductase-1 mRNA was not detected in heart and muscle tissues. As a result, the mRNA used as control samples was isolated from liver (positive control) and heart (negative control) tissues.



FIGURE 4.1. Expression of 5 α -R1 mRNA in rat tissues. 5 α -R1 expression in rat tissues was analysed by RT-PCR followed by electrophoresis on a 2% agarose gel. With the exception of the testis, total RNA was isolated as described (Chomczynski and Sacchi, 1987) from the indicated tissues collected from 21-day old (immature) female Wistar rats. Ovarian and uterine RNA was isolated from (C) immature untreated rats (controls), (P) immature rats injected with single dose of eCG (10 iu) for 48 hours, and (H) immature rats injected with eCG (as explained) followed by a single dose of hCG (10 iu) for 12 hours. Testicular RNA was isolated from adult male Wistar rats. Nuclease-free water (H₂O) was used as a negative control for the PCR reaction.

4.3.1.2. Expression of 5 β -reductase in rat tissues

5 β -Reductase mRNA was not expressed in rat ovary or muscle, but was abundant in the liver (Fig. 4.2).

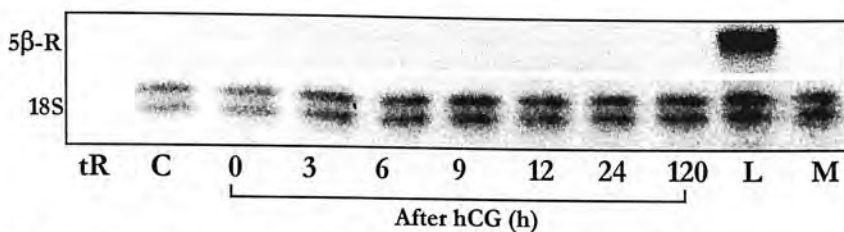


FIGURE 4.2. Expression of 5 β -reductase (5 β -R) mRNA in rat ovaries. The autoradiogram (see "Experimental Procedures, 4.2.5") shows the expression of 5 β -R mRNA analysed by RPA of total RNA (20 μ g) isolated from rat ovaries collected from controls, immature untreated rats (C), and rats treated with a single injection of 10 IU eCG for 48 h followed by a single injection of 10 IU hCG for 0, 3, 6, 9, 12, 24, 120h. Female rat liver (L) and muscle (M) were positive and negative controls, respectively. Transfer RNA (tR) was used as the negative control for the assay. (18S - ribosomal RNA)

4.3.2. Effect of gonadotrophins on 5 α -R1 mRNA expression in rat ovary

To study the effect of gonadotrophic stimuli on ovarian 5 α -R1 expression, total RNA isolated from whole ovaries collected from rats stimulated for different periods of time, was analysed by RPA.

The expression of 5 α -R1 mRNA was highest in immature, unstimulated ovaries (controls) as shown in Fig. 4.3. The expression was down-regulated after treatment with eCG to levels that were about 20-30% of the control value. Subsequent treatment with hCG caused an initial drop in the levels of 5 α -R1 to 10% of the control levels during the first six hours after which hCG gradually stimulated 5 α -R1 mRNA expression. The increase in 5 α -R1 mRNA expression continued to increase 12 h after hCG treatment and was highest after 24 h when it had returned to 80% of the control value. However, the hCG-stimulatory effect was transient because 5 α -R1 mRNA expression had decreased to almost 10% of the control levels at 120 h after hCG treatment. Because 5 α -R1 mRNA expression was re-expressed at 12 h and 24 h after hCG, the time-points were used in subsequent experiments to study the effects of eCG and hCG on the expression of 5 α -R1 in rat ovaries during preovulatory development.

4.3.3. Preparation of Plasmid 5 α -R1 cDNA

4.3.3.1. Synthesis of 5 α -R1 cDNA by RT-PCR

A 5 α -R1 PCR product sized between 396 and 506 bp, encompassing the target construct size (~456 bp), was detected in the rat liver samples and ovary, but none was present in distilled water (Fig.4.4). There was no genomic DNA contamination in the amplified 5 α -R1 cDNA.

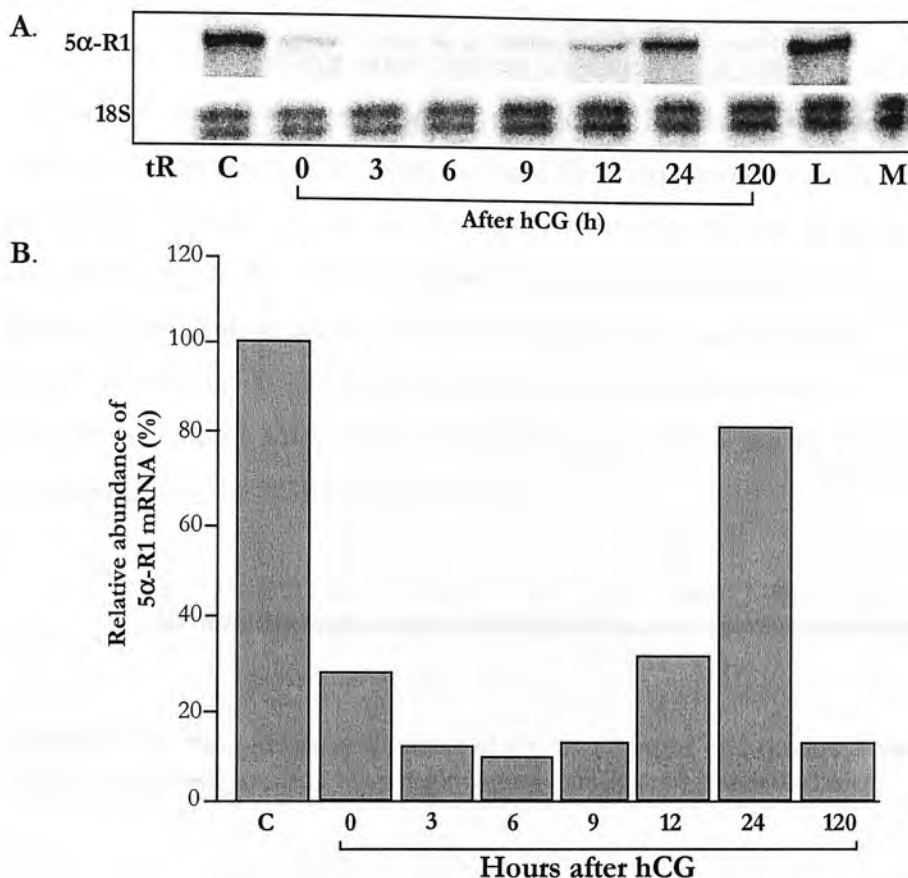


FIGURE 4.3. Expression of 5 α -reductase type 1 in rat ovaries. **A.** The autoradiogram of RPA products (see "Experimental Procedures, 4.2.5") shows the expression of 5 α -R1 mRNA in rat ovaries collected from controls, immature untreated rats (C), and rats treated with a single injection of 10 IU eCG for 48 h followed by a single injection of 10 IU hCG for 0, 3, 6, 9, 12, 24, 120 h. Female rat liver (L) and muscle (M) were positive and negative controls, respectively. Transfer RNA (tR) was used as the negative control for the assay. **B.** Quantitative analysis of 5 α -R1 expression shows the levels of 5 α -R1 mRNA in the described groups. The abundance of 5 α -R1 mRNA was normalised using ribosomal RNA (18S) and expressed as percentage of the control value.

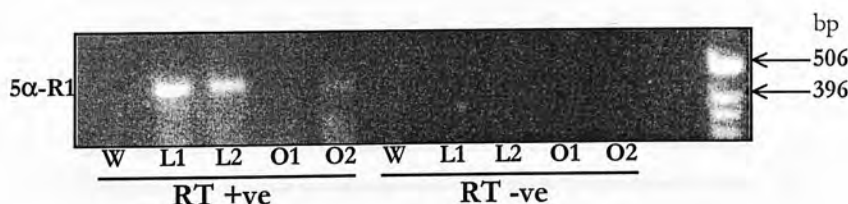


FIGURE 4.4. Preparation of 5 α -R1 cDNA. Duplicate aliquots of total RNA isolated from immature female rat liver and ovary, liver 1 (L1) and 2 (L2), and ovary (O1) and (O2), respectively, were amplified by reverse transcription-polymerase chain reaction (RT-PCR) as described under "Experimental Procedures, 4.2.3.1." Distilled water samples (W) were used as negative control samples. The PCR was performed after RT (RT +ve) and without RT (RT -ve) to analyse genomic DNA contamination. The sizes of the constituent fragments of the 1 Kb DNA ladder (GIBCO, Cat. No. 15615-016) are indicated on the right side of the gel.

4.3.3.2. Cloning of 5α-R1 cDNA

The 5α-R1 PCR product (probe), containing a *Pst* I cleavage site, was cloned successfully into pBluescript II in a 5' - 3' (sense) orientation that was identified by cleavage of the plasmid cDNA with *Pst* I and direct sequencing. Fig. 4.5 shows a sketch of the 5α-R1 probe and the site at which *Pst* I cleaves the 5α-R1 DNA probe. The identity of the 5α-R1 probe, confirmed by a blast search at the National Centre for Biotechnology Information (NCBI; Bethesda, MD), was shown to have 100% homology with rat 5α-R1 mRNA gene, which corresponds, to loci 10 - 777 of the complete rat 5α-R1 gene. Fig. 4.6 shows the sequence of the rat 5α-R gene that is encompassed by the 5α-R1 cDNA probe.

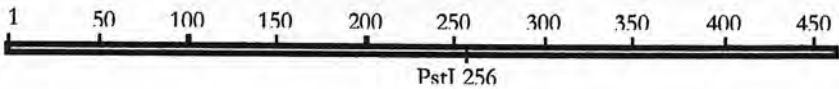


FIGURE 4.5. Graphic representation of the 5α-R1 probe and the *Pst* I restriction site. The restriction enzyme, *Pst* I has a single cleavage site (256 nt) within the probe.

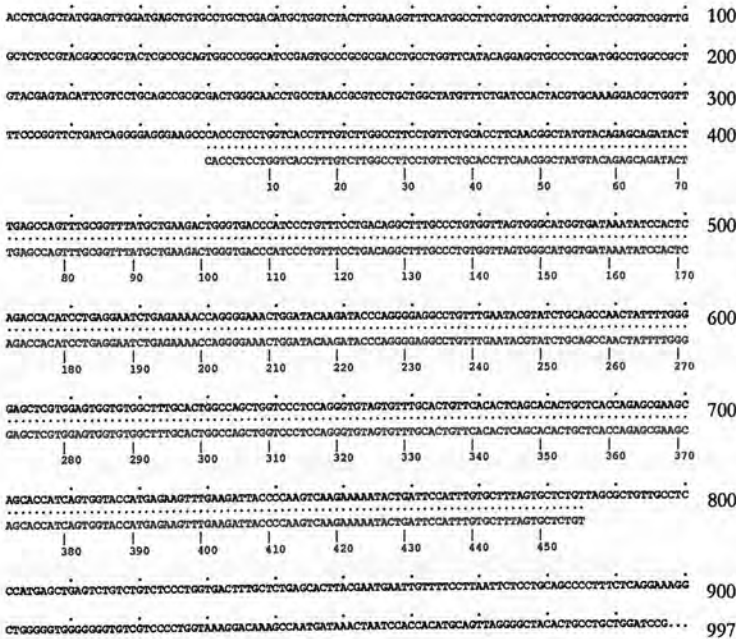


FIGURE 4.6. Nucleotide sequence of cloned rat 5α-R1 insert aligned with the rat 5α-R1 gene. The verification of the sequence of the cloned 5α-R1 plasmid DNA by Blast searching at the National Centre for Biotechnology information (NCBI; Bethesda, MD) revealed that the insert (456 bp; red font) had 100% homology with rat 5α-R1 gene (black font; Anderson et al., 1989). The insert represented the amino acids 330-785 of the complete rat 5α-R1 gene. The nucleotides in 5α-R1 gene are numbered on the left. The black dots represent every tenth nucleotide.

4.3.4. Expression of 5 α -R1 and aromatase mRNA in rat ovary during the preovulatory period.

To show the expression of 5 α -R1 and aromatase mRNA in specific tissues in the rat ovary, total RNA isolated from granulosa, residual and whole ovary was analysed by northern analysis.

4.3.4.1. Comparison of 5 α -R1 and aromatase mRNA expression in granulosa cells and residual ovary.

The expression of 5 α -R1 mRNA was more abundant in residual ovary tissue than the granulosa cells (Fig. 4.7). The gene expression was highest in the residual ovary of immature untreated rats and was significantly reduced to 35% of the control value by treatment with eCG ($P < 0.001$). Subsequent treatment with hCG for 12h induced a transient increase in the expression of 5 α -R1 mRNA expression to 60% of the control value. At 24h after hCG treatment, 5 α -R1 mRNA had decreased significantly to 40% of the control value ($P < 0.001$). Due to the evidence in Chapter 5, the similar pattern of expression of lower levels of 5 α -R1 mRNA in granulosa cells is due to contamination of granulosa cells with theca/interstitial cells. The expression of 5 α -R1 mRNA in the residual ovary of DES-treated rats was similar to that in the control animals. The liver expressed very high levels of 5 α -R1 mRNA, but the gene was absent in the heart tissue.

Re-hybridising the RNA blots shown in the top panel of Fig. 4.7 with aromatase riboprobes showed that the aromatase probe hybridised to three mRNA transcripts (3.3, 2.6 and 1.9 Kb in size) in rat ovarian tissues (Fig. 4.7, middle panel). The most abundant transcript was the 3.3 Kb band. It was expressed in granulosa cells, and was essentially absent in the residual ovary tissue. The aromatase mRNA transcripts, particularly the 3.3 Kb and 1.9 Kb bands, were most abundant in the eCG-treated granulosa cells. Both transcripts were detected in the granulosa cells from immature and hCG-treated rats, but at levels lower than in eCG-treated granulosa cells. The faint signal detecting the 3.3 Kb aromatase mRNA transcript in the residual ovary of eCG-treated rats was due to contamination of the residual ovary tissue with granulosa cells. The 1.9 Kb transcript, evidently expressed in the granulosa cells and less so in the residual ovary, was also expressed in the heart and liver tissues. However, the 3.3 Kb and 2.6 Kb transcripts were not expressed in residual ovary of immature, hCG-, DES-treated rats, or heart and liver tissue.

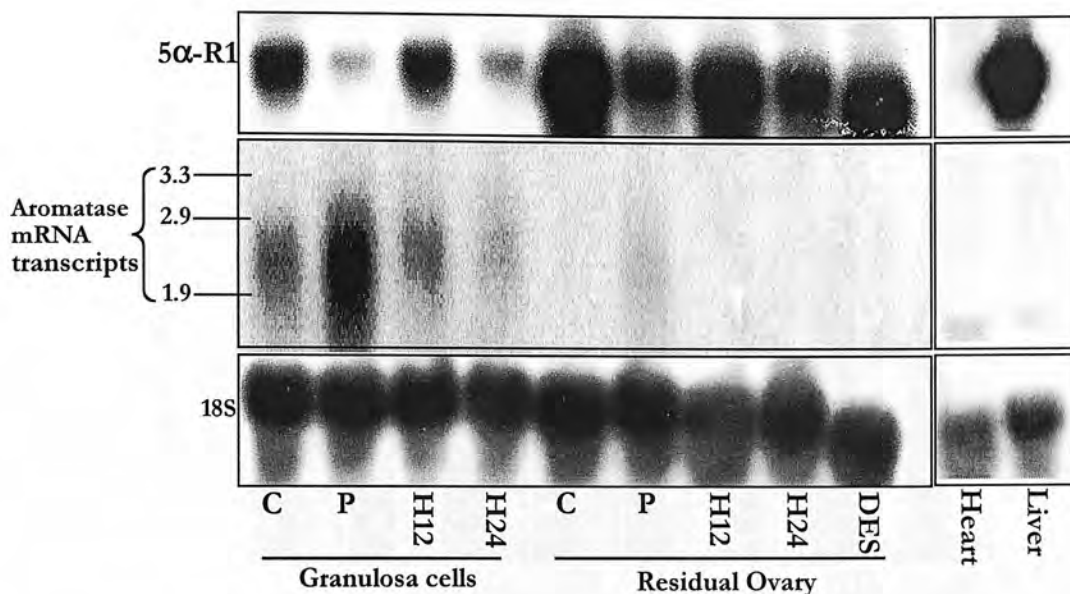


FIGURE 4.7. Expression of 5 α -R1 and aromatase mRNA in rat ovarian tissues. Northern blots of total RNA (20 μ g) isolated from ovarian tissues of rats treated with hormones as described (Experimental Procedures, 3.2.1) showed the expression of 5 α -R1 (*top panels*) and aromatase (*middle panels*) mRNA in granulosa cells and residual ovarian tissue in immature untreated rats, controls (C), eCG-treated rats (P), rats injected subsequently with hCG for 12 h (PH12) and 24 h (PH24). In addition, 5 α -R1 mRNA expression was detected in diethylstilboestrol (DES)-treated residual ovary. RNA isolated from heart and liver tissue was included as negative and positive control material, respectively. The blots were prepared as described (NorthernMax Northern Blotting Kit, Ambion Cat. No. 1940) and analysed by autoradiography after overnight exposure to Kodak X-ray film at -70°C . 5 α -R1 and aromatase mRNA were hybridised with ^{32}P -labeled riboprobes prepared by *in vitro* transcription of PCR-generated cDNAs specific to 5 α -R1 and aromatase genes, respectively. The aromatase probe hybridised to three mRNA transcripts, 1.9 Kb, 2.9 Kb and 3.3 Kb in size. Ribosomal RNA (18S) was hybridised by ^{32}P -labeled DNA probes (*bottom panels*).

ECG and hCG regulated aromatase and 5 α -R1 mRNA in the rat ovary in an inversely related pattern during preovulatory development (Fig. 4.8). While eCG down-regulated 5 α -R1 mRNA expression in residual ovary, it up-regulated aromatase mRNA expression in granulosa cells significantly ($P < 0.05$), and whereas hCG up-regulated 5 α -R1 mRNA expression transiently, it had no effect on aromatase expression.

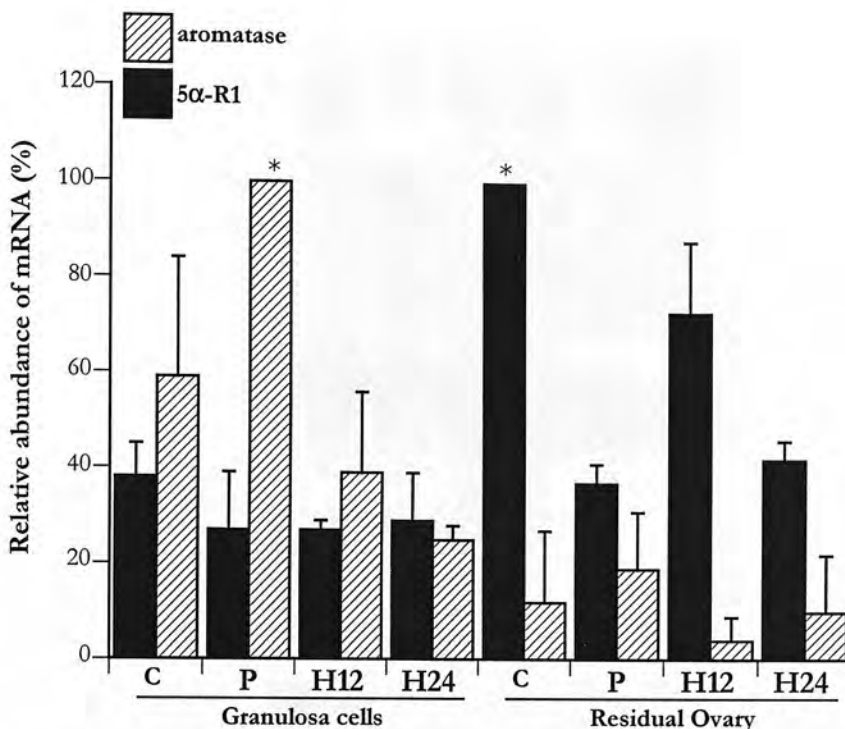


FIGURE 4.8. Inverse relationship between 5α-R1 and aromatase mRNA expression during preovulatory development. Quantitative analysis of the radioactive signals of 5α-R1 and aromatase mRNA performed as described (Experimental Procedures, 4.2.7), shows the patterns of each mRNA expression in preovulatory rat ovary. C= control; P= eCG-treated rats; (PH12)= rats injected subsequently with hCG for 12 h; (PH24) = rats injected subsequently with hCG for 24 h. Each bar represents mean \pm SD percentage of relative abundance of mRNA in 3 replicate experiments. Asterisks denote significant increase ($P < 0.05$) in gene expression compared to other groups.

4.3.4.2. Comparison of 5α-R1 and aromatase mRNA expression in whole ovary

The expression of rat 5α-R1 and aromatase mRNA in whole ovary was clearly defined (Fig. 4.9). The abundant expression of 5α-R1 mRNA gene in immature ovary was reduced by treatment with eCG to levels that were non-detectable by northern analysis while eCG stimulated aromatase mRNA expression. The aromatase gene (all three mRNA transcripts) was only detected in the eCG-treated ovary where it was expressed abundantly. Aromatase mRNA was not detected in the immature and hCG-treated ovaries. Subsequent treatment with hCG for 12h restored a transient increase in 5α-R1 mRNA gene expression, which was decreased after 24h to levels that were higher than in the eCG-treated ovaries. Conversely, hCG did not affect aromatase mRNA expression.

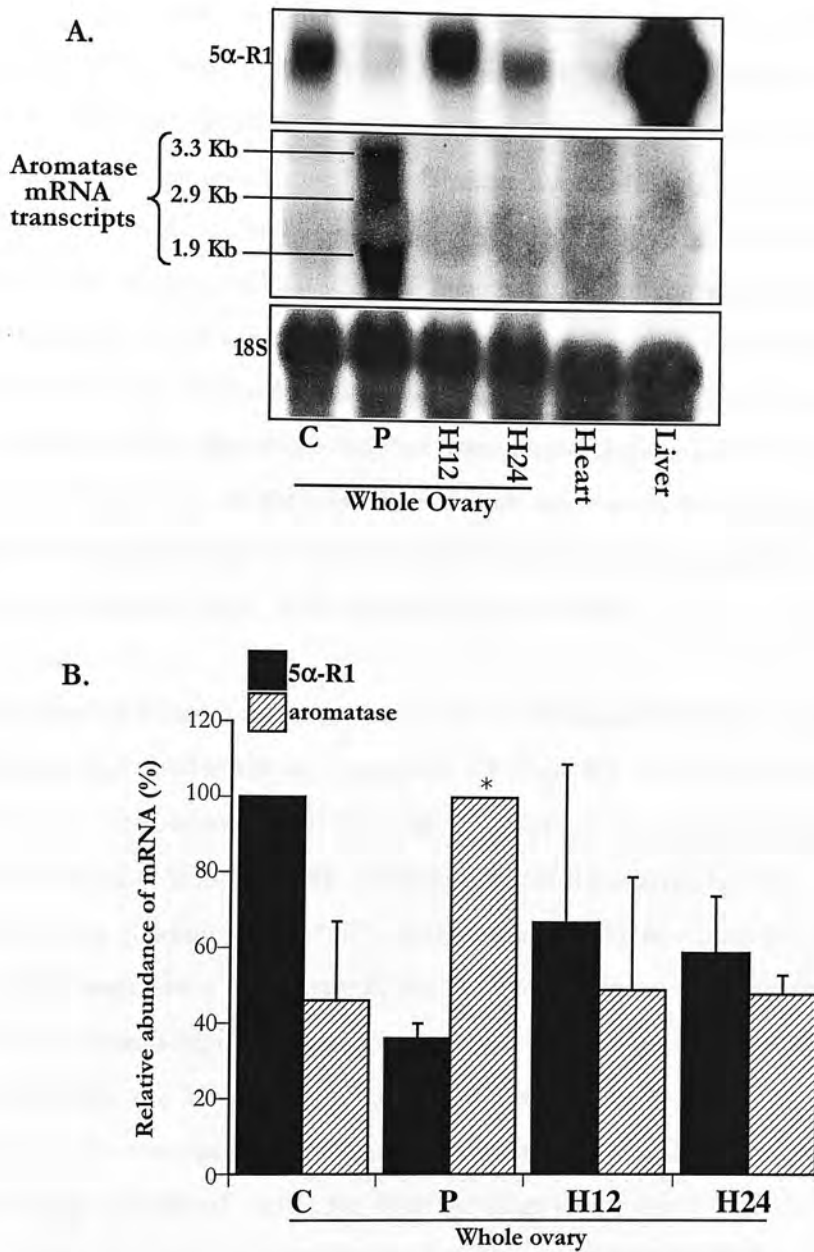


FIGURE 4.9. Relative expression of 5 α -R1 and aromatase mRNA in whole ovarian tissue. **A.** Northern blots of 5 α -R1 (*top panel*) and aromatase (*middle panel*) mRNA in whole ovaries that were harvested from rats treated with hormones as described (Experimental procedures, 3.2.1) were set up with 20 μ g total whole ovary RNA as described (NorthernMax Northern Blotting Kit, Ambion, UK, Cat. No. 1940). The indicated mRNA in the ovaries of immature untreated rats, controls (C), eCG-treated rats (10 iu for 48 h) (P), and rats subsequently injected with hCG (10 iu) for 12 h (PH12) and 24 h (PH24) was hybridised with specific 32 P-labeled riboprobes generated by *in vitro* transcription as described (MAXIscript *In vitro* Transcription Kit, Ambion, Cat. No. 1324). The sizes of the three transcripts of aromatase mRNA are shown alongside the gel. H- heart; L- liver; 18S- ribosomal RNA. **B.** Quantitative analysis of whole ovary 5 α -R1 and aromatase mRNA was normalised using 18S rRNA and expressed as a percentage of the control and eCG-treated value, respectively. Each bar represents mean \pm SD percentage of relative abundance of mRNA in 3 replicate experiments. Asterisks denote significant increase stimulation ($P < 0.05$).

4.4. DISCUSSION

It is well documented that the metabolism of ovarian androgens is central to the normal function of the ovary (Hillier, 1985; Adashi, 1994; McGee & Hsueh, 2000). In addition to aromatase as the main ovarian enzyme, minor enzymes such as 5 α -R also convert androgens into alternate metabolites (Eckstein *et al.*, 1970; Lerner & Eckstein, 1976; McNatty *et al.*, 1981; Gore-Langton & Armstrong, 1988). The different steroids have distinct roles during follicular development and, hence the expression of the enzymes responsible for their metabolism is tightly regulated as the ovary undergoes the developmental changes (Richards, 1994). It has been reported that the enzymes, aromatase and 5 α -R have opposing effects on follicular development in the adult ovary (Lephart *et al.*, 1992). To study the regulation of both enzymes in the studies described in this chapter, the gonadotrophic control of the enzymes was investigated in ovaries of immature rats, the animal model of choice used in these studies.

5 α -Reductase exists as two isozymes (Russell & Wilson, 1994), but it was shown that the isozyme expressed in the rat ovary is 5 α -R1 (Figs. 4.1, 4.3 and 4.4) and not 5 β -R (Fig. 4.2). The 5 α -R isozyme also exists as either type 1 or type 2 (Normington & Russell, 1992; Russell & Wilson, 1994). Although the 5 α -R2 enzyme has been reported in the human ovary (Jakimiuk *et al.*, 1999; Akahira *et al.*, 2001), no conclusive evidence illustrates 5 α -R2 expression or activity in the rat ovary to date. During the studies described in this manuscript, attempts to develop methodologies to illustrate 5 α -R2 expression alongside the 5 α -R1 studies were unsuccessful due to failure to develop a 5 α -R2 cDNA probe corresponding to a specific sequence of 5 α -R2 gene as clarified by BLAST searching at National Centre for Biotechnology information (NCBI; Bethesda, MD). The riboprobes used to hybridise the 5 α -R1 gene (either by RPA or northern analysis) detected a single mRNA species approximately 2.5 Kb in size and homologous with the 5 α -R1 mRNA detected previously in rat liver (Andersson *et al.*, 1989). The findings showed that the enzyme was expressed selectively in various rat tissues including the ovary (Fig. 4.1) and that the expression of ovarian 5 α -R1 mRNA is regulated by both gonadotrophins, FSH and LH (Figs 4.3, 4.7 & 4.9).

Specific gonadotrophic regulation of 5 α -R1 mRNA expression was shown by the *in vivo* studies illustrated in Fig. 4.3. Equine CG suppressed 5 α -R1 expression which

was transiently restored by hCG indicating opposing roles of the pituitary hormones on ovarian 5 α -R1 expression. In earlier studies, (Aono *et al.*, 1981) showed that FSH had an inhibitory effect on 5 α -R1 activity and the results suggest that the effect occurs at the gene level where the gonadotrophin downregulates 5 α -R1 mRNA expression (5 α -R1 panels; Figs. 4.7 & 4.9).

Knowing that ovarian tissues respond essentially to either gonadotrophin, northern analyses of granulosa cells and residual ovary tissue which was enriched in theca/interstitial cells showed that 5 α -R1 mRNA was expressed more abundantly in residual tissue than granulosa cells (Fig. 4.7). The expression of the gene in the residual ovary was highest in ovaries of immature rats and was reduced by eCG, but increased again by hCG, albeit transiently. A similar trend was observed in the granulosa cells, but this was a result of contamination of granulosa cell preparations by theca/interstitial material, and localisation studies described in later studies (Chapter 5) clarify the tissue specificity of 5 α -R1. The response of 5 α -R1 mRNA to hCG was also highly indicative of the enzyme's location in thecal/interstitial cells because LH action is mediated through receptors that are located on theca cells in immature ovaries. However, eCG (which is FSH-like in action) decreased the expression of 5 α -R1 mRNA. FSH action is mediated through granulosa cells because they, unlike theca cells, express FSH receptors. This suggests that down-regulation of 5 α -R1 mRNA by FSH must involve other FSH-induced granulosa cell factors that decrease 5 α -R1 expression. These paracrine modulators of 5 α -R1 expression are yet to be identified.

In contrast to 5 α -R1 expression, ovarian aromatase expression was upregulated by eCG and downregulated by hCG. Similarly to the well-documented regulation of aromatase by FSH (Hillier, 1985; Adashi, 1994; Richards, 1994; McGee & Hsueh, 2000), the almost negligible levels of aromatase mRNA detected in granulosa cells of immature ovaries were increased by eCG treatment *in vivo* (aromatase panels; Figs. 4.7 & 4.9). Human CG suppressed the eCG stimulatory action on the expression of aromatase mRNA. The opposing effects of eCG and hCG on the regulation of ovarian 5 α -R1 and aromatase were also described by Lephart *et al.*, (1992). Whether in specific ovarian cell types (Figs. 4.7 & 4.8) or whole ovary (Fig. 4.9), the inverse relationship of the expression of 5 α -R1 and aromatase suggested that that during preovulatory

development the stimulatory action of FSH on aromatase expression is upregulated when the expression of 5 α -R1 is inhibited. Likewise, 5 α -R1 expression is stimulated by LH when aromatase is inhibited suggesting that 5 α -reductive metabolism is a favoured pathway when aromatase activity, (i.e. oestrogen production) is suppressed. Since oestrogens are required for follicular maturation, it is likely that 5 α -reduced steroids are only synthesised when follicular development must be suppressed as in ovaries of immature animals. This is possibly why 5 α -R1 mRNA is highly elevated while aromatase is hardly expressed in the ovaries of immature rats (as shown in Figs. 4.7 & 4.9) so as to prevent precocious secretion of oestrogens. Infact, 5 α -R1 activity has been shown to be highly elevated in ovaries of immature rats (Inaba *et al.*, 1978; Fukuda *et al.*, 1979; Aono *et al.*, 1981). In adult animals, as follicular development progresses mainly due to FSH, the over riding requirement for FSH results in an upregulation of aromatase expression which causes oestrogen production (Richards, 1994). The expression of 5 α -R1 and hence 5 α -reductase action, is simultaneously suppressed so that the inhibitory effect of 5 α -reduced androgens (Hillier *et al.*, 1980a; Conway *et al.*, 1990) is removed.. The net consequence is enhanced synthesis of oestrogens required to complete follicular maturation leading to ovulation. The studies described in this chapter have shown that the expression of 5 α -R1 mRNA is transiently upregulated by eCG (Figs. 4.3, 4.7 & 4.9) suggesting roles for 5 α -reduced steroids during the periovulatory period. Which 5 α -reduced steroids are present at this stage and what their functions are, are still largely unknown. It seems more likely that the substrate metabolised by 5 α -R1 is progesterone, which is the steroid produced increasingly during periovulation

Equine CG clearly reduced 5 α -R1 mRNA expression, which was restored by hCG, indicating down- and up-regulating effects of FSH and LH, respectively, on the 5 α -R1 gene (Fig. 4.3). Others have reported similar findings of a stimulatory effect of LH (Aono *et al.*, 1981) and an inhibitory effect of FSH (Aono *et al.*, 1981; Payne *et al.*, 1992). Since FSH levels begin to rise during the early stages of preovulatory follicle growth, it implies that the expression of 5 α -R1 is inhibited correspondingly during this stage. Physiologically, the gradual inhibition of 5 α -R1 expression removes the inhibitory effect of 5 α -R1 on oestrogen synthesis. It is known that oestrogens are secreted in increasing amounts during preovulatory follicle development and are crucial

for ovulation (Greenwald & Terranova, 1988; Adashi, 1994; McGee & Hsueh, 2000). The one or few recruited follicles, in monovulatory species or multi-ovulatory species, respectively, that become the dominant follicles require high levels of oestrogens which stimulate their further growth. As FSH-induced oestrogen secretion increases, the oestrogenic output eventually down-regulates the release of pituitary FSH by negative feed back. The removal of any impediment to oestrogen production is therefore critical to the developmental progress of healthy preovulatory follicles. 5 α -reduced androgens inhibit ovarian oestrogen biosynthesis by competitive inhibition of aromatase (Hillier *et al.*, 1980a). The down-regulation of 5 α -R1 by FSH and hence, 5 α -reduced androgens is probably part of the intraovarian mechanisms which ensure that the inhibition of oestrogen production is removed during the preovulatory stages when the developing follicles increasingly depend on oestrogen for full maturation.

As the dominant follicle(s) approaches ovulation, the levels of LH begin to peak hours before ovulation takes place. The transient stimulatory effect of hCG on 5 α -R1 mRNA *in vivo* which occurred after hCG administration (Fig. 4.3, 4.7, 4.8, 4.9) suggests that the peri-ovulatory LH surge stimulates 5 α -R1 expression around the time of ovulation. As mentioned above, it is not yet clear why 5 α -reductive metabolism is required around ovulation, but it might involve metabolism of progesterone, which is the main ovarian hormone after ovulation. Since 5 α -R1 metabolises progesterone to other less biologically active forms (Verma & Laumas, 1976; Frye & Vongher, 2001) and progesterone is a better substrate for 5 α -R1 than androgens (Payne *et al.*, 1992), it is probably metabolised readily during the peri-ovulatory phase indicating a role of 5 α -reduced progestins during this stage of ovarian function. According to Eckstein *et al.*, (1970), these steroids might be excretory intermediates, suggesting that the role of 5 α -R1 at this stage is to contribute to the excretion of excess progesterone. Or it could be that 5 α -R1 action is required during conditions of high concentrations of progesterone suggesting that the enzyme might play an unknown role during pregnancy.

Although the studies described in this chapter indicated that rat ovarian 5 α -R1 mRNA gene is expressed in residual ovary and not granulosa cells, the cellular localisation was still indistinct. This was clarified in the next chapter.

Chapter 5. Cellular localisation of 5 α -reductase type 1 in the rat ovary

5.1. INTRODUCTION.

5 α -reductase type 1, the 5 α -R isozyme present in rat ovary is a gonadotrophically-regulated enzyme that modulates follicle development via control of oestrogen production. Although the ovarian expression of the enzyme has been clearly demonstrated in immature (Eckstein & Nimrod, 1977) and adult ovary (Lephart *et al.*, 1992; Haning *et al.*, 1996), the specific cellular sites of 5 α -R1 in the ovary are still unknown. Most recent studies of the enzyme in the ovary have investigated its activity and gene expression (Lephart *et al.*, 1992; Haning *et al.*, 1996; Jakimiuk *et al.*, 1999) in relation to oestrogen production, but have not specified its cellular localisation. Studies of the cellular localisation of 5 α -R1 have largely focused on male reproduction (Pelletier *et al.*, 1998) or non-reproductive tissues such as liver (Eicheler *et al.*, 1995), nervous system (Poletti *et al.*, 1997; Yokoi *et al.*, 1998a), adrenal gland (Yokoi *et al.*, 1998b) and skin (Bayne *et al.*, 1999; Thiboutot *et al.*, 2000) where the enzyme is crucial for normal development and/or function of these organs.

In the previous chapter, high levels of 5 α -R1 mRNA gene were detected in residual ovarian tissue compared to isolated granulosa cells suggesting that the cellular location was principally confined to the thecal/interstitial compartment of the ovary. The indication of such a polarised distribution of 5 α -R1 to thecal/interstitial cells, and not granulosa cells, is reinforced by the increased activity of 5 α -R1 in residual ovary compared to granulosa cells, in response to LH stimulation, whereas FSH is inhibitory (Fukuda *et al.*, 1979; Payne *et al.*, 1992). In addition, the stimulatory effect of LH on both 5 α -R activity and proliferation of interstitial cells, with no such effect on granulosa cells (Aono *et al.*, 1981), strongly suggests that the expression of the enzyme is specific to thecal/interstitial tissue other than granulosa cells. Verification of the cellular location of 5 α -R1 in the ovary is important in identifying the specific sites influenced by gonadotrophic signals, and therefore, could be used to develop therapies which target specifically 5 α -R1 in disease conditions where the enzyme's activity is dysfunctional.

In this study, the cellular distribution and regulation of ovarian 5 α -R1 during preovulatory follicle development was studied by investigating the distributions of both 5 α -R1 protein and mRNA gene. Most importantly, a novel finding is presented showing distinct polarisation of the cellular localisation of 5 α -R1 in thecal/interstitial tissue while absent in granulosa cells in the rat ovary.

5.2. EXPERIMENTAL PROCEDURES

5.2.1. Wax-embedded tissues

Ovarian tissues were obtained from animals given *in vivo* hormonal treatments as previously described (Chapter 3, section 3.2.1). Heart and skeletal muscle tissues were obtained from control rats. The tissues were fixed in either Bouins fixative (Triangle Biomedical Sciences, Skernersdale, UK) for 6 h or normal buffered formaldehyde (NBF) for 24 h. Fixed tissues were immersed in 70% ethanol until further processing for embedding in paraffin wax.

5.2.2. Immunocytochemical procedures

Serial sections of paraffin-embedded tissues (5 μ m) fixed in Bouins or NBF-fixed tissues were mounted on slides (Superfrost Plus, BDH, Cat. No. 406/0179/00), dried overnight at 37°C, dewaxed in xylene and rehydrated. Endogenous peroxidases were blocked with 9% hydrogen peroxide in methanol for 30 min. After subsequent 5-min washes in water and Tris-buffered saline (TBS), the sections were incubated for 30 min at room temperature with normal porcine serum (NSS) prepared as a solution of 1 ml normal swine serum and 5% bovine serum albumin (BSA) in 4 ml TBS. Following the BSA block, the porcine serum was removed and immediately replaced with 5 α -R1 antiserum (Eichele *et al.*, 1995), diluted 1:500 and 1:100 in TBS for tissues fixed in Bouins and NBF, respectively. Negative control sections were treated with similarly diluted non-immune rabbit serum instead of 5 α -R1 antiserum. The immunoreaction was incubated overnight at 4°C. After two 5-min washes in TBS, biotinylated swine anti-rabbit (DAKO, Denmark, Cat No. E 0353), diluted 1:500 in NSS, was added on to the sections and incubated for 30 min at room temperature. After two 5-min washes in TBS, ABC-HRP complex (DAKO, Denmark, Cat. No. K0355) in salt-free Tris buffer (0.05M, pH 7.4) was added to the sections. Tissues were incubated for 30 min at room

temperature. Two subsequent 5-min washes in TBS were followed by addition of the chromagen, 3,3-diaminobenzadine tetrahydrochloride (DAB) which was prepared as described (DAKO, Denmark, Cat. No. K3468). Specific immunostaining was localised as brown pigmentation. The colour development reaction was stopped by immersing the slides in water. The sections were subsequently dehydrated, counterstained with haematoxylin (see Appendix I), mounted in resin, and left to dry before analysis by light microscopy.

5.2.3. In situ hybridisation

Serial sections of paraffin-embedded tissues (5 μ m) fixed in Bouins were mounted on slides, dried overnight at 37°C. *In situ* hybridisation was adapted from methods described by Slee *et al.*, (2001). Prehybridisation was carried out by dewaxing the tissues in histoclear, rehydrating, refixing, and permeabilising by digestion with proteinase K at a concentration of 10 μ g/ml for 7.5 min at 37°C. Followed by subsequent washes in PBS and 2X SSC, the tissues were then acetylated and rewashed in 2X SSC prior to dehydration through a series of graded alcohols. Hybridisation was performed overnight at 55°C with [³⁵S]UTP-labelled complimentary RNA (cRNA) probes (antisense riboprobes) generated from cDNA templates described in Chapter 4, Experimental procedures, 4.2.4.2. I. using an RNA transcription kit (Promega Corp.). Sense riboprobes, used as controls for non-specific binding, were generated similarly from cDNA cleaved with *Not* I instead of *Eco*R V. Post hybridisation washes were done in buffers of decreasing salt concentrations at 55°C, followed by dehydration through ethanol gradients, and processed for liquid emulsion autoradiography (Kodak NTB-2). After exposure for 3 weeks at 4°C, the slides were developed and counterstained with haematoxylin for photomicrography.

5.2.4. Data Analysis

The specific 5 α -R1 immunostaining and radioactive signals were quantified as optical densities and granules per unit area, respectively, by image analysis (Image Pro-Plus software, Media Cybernetics, L.P., Silver Springs, MN, USA). The abundance of 5 α -R1 protein and mRNA was expressed as a percentage of the control values. Data were analysed by one-way ANOVA, using Student's t-test to identify significant differences between treatments.

5.3. RESULTS

5.3.1. Cellular Localisation of 5 α -R1 in Immature Rat Ovary

Using ovarian tissue sections of immature, untreated rat ovary, the cellular localisation of 5 α -R1 protein and mRNA gene was investigated using immunocytochemistry (ICC) and *in situ* hybridisation (ISH), respectively.

5.3.1.1. Localisation of 5 α -R1 in rat ovary by immunocytochemistry

Thecal and interstitial cells showed strong and specific immunostaining with the 5 α -R1 antiserum, but the granulosa cells and ovarian stromal tissue were devoid of any immunoreactivity (Figs. 5.1A, C and 5.2). No immunoreaction occurred in corresponding tissue sections that were incubated with non-immune rabbit serum instead of the anti-serum (Fig. 5.1B and D). The specific immunostaining in ovaries fixed in either Bouins or NBF was localised to thecal/interstitial tissue. The oocytes in Bouin's-fixed tissues had slight non-specific immunostaining (Fig. 5.1A) which was absent in NBF-fixed tissues (Fig. 5.1C) or *in situ* (Fig. 5.4A). Figure 5.2 shows a high power image of the cellular distribution of 5 α -R1 in immature rat ovary fixed in Bouins.

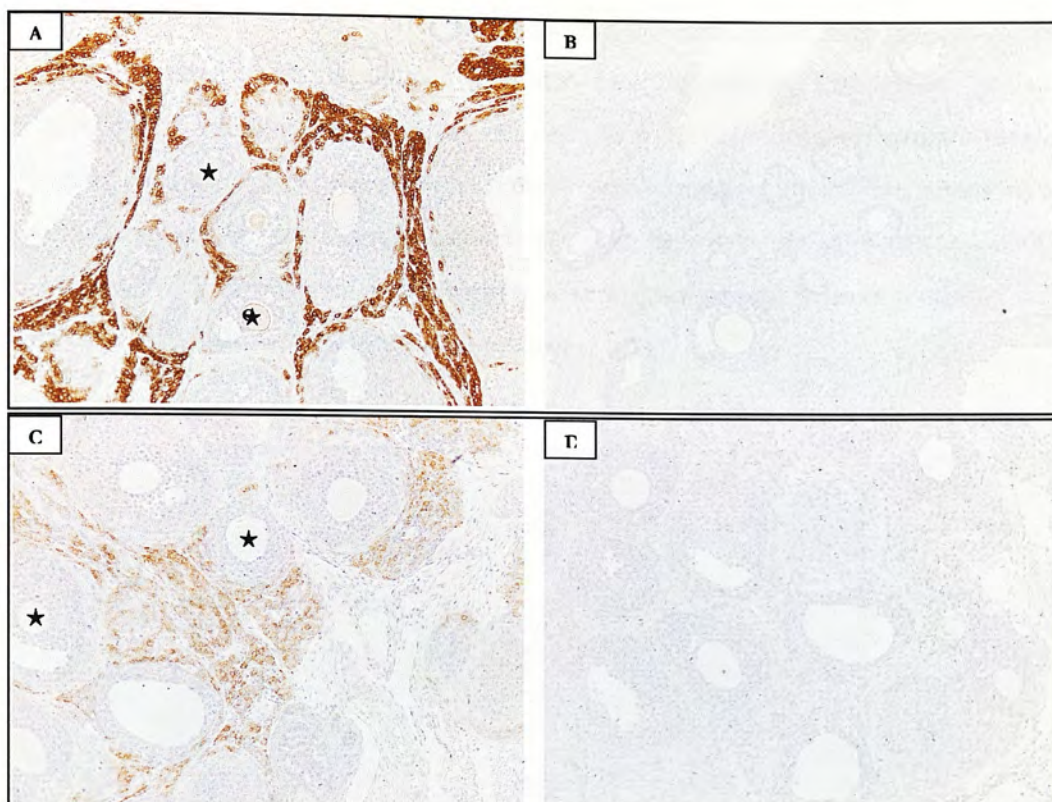


FIGURE 5.1. Cellular localisation of 5 α -R1 in immature rat ovaries fixed in Bouins and NBF. The cellular localisation of 5 α -R1 in ovaries (see "Experimental Procedures, 5.2.2) fixed in Bouins (top panel) and NBF (bottom panel) was done in tissues incubated with 5 α -R1 antiserum (A & C) and non-immune rabbit serum (B & D). Non-specific immunostaining of the oocytes (★) in the Bouins-fixed tissue was not reflected in NBF-fixed ovarian tissue.

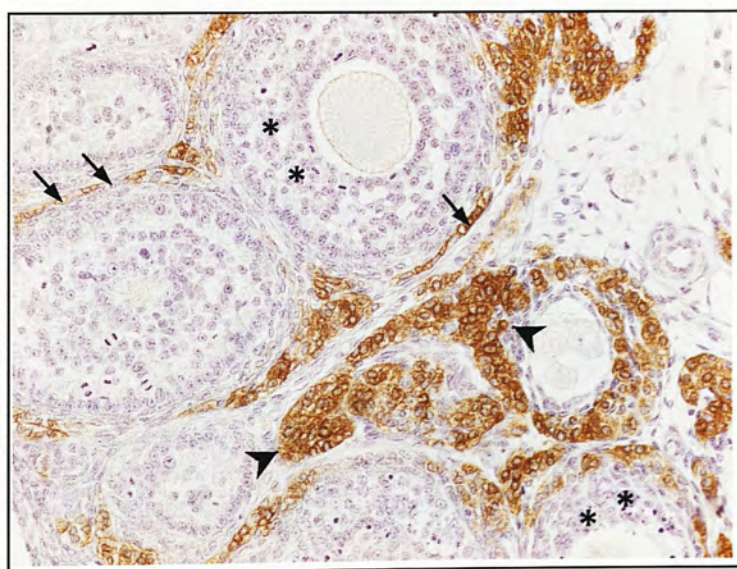


FIGURE 5.2. High power image of specific 5 α -R1 immunostaining in immature rat ovary. Specific 5 α -R1 immunostaining in thecal (solid arrows) and interstitial (arrow heads), while absent in granulosa cells (asterisks), shows the enzyme's cellular distribution in ovaries fixed in Bouins.

The heart (negative control tissue) showed no immunoreactivity (Fig. 5.3A), but the liver (positive control tissue) had very intense immunostaining throughout the tissue (Fig. 5.3B). The immunoreaction was only present in the hepatocytes where the staining intensity showed a gradient pattern with more intense staining around the portal canals which decreased radially into the hepatocytes. Connective tissue cells were unstained. Consistent with the ovarian tissue, immunostaining was absent in liver tissue sections that were incubated with non-immune serum.

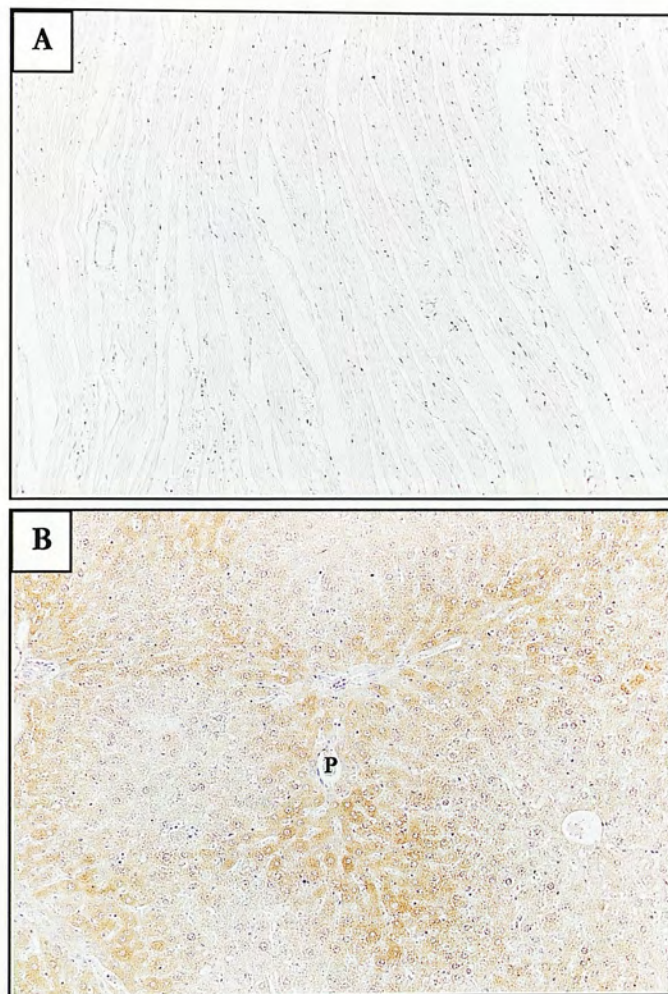


FIGURE 5.3. Immunocytochemical staining of 5 α -reductase type 1 in immature rat heart and liver. As described (Experimental Procedures, 5.2.2), localisation of 5 α -R1 was investigated in Bouins-fixed heart (A) and liver (B) tissues collected from immature female rats. Inset panels- tissues incubated with non-immune serum; P- portal canal.

5.3.1.2. Localisation of 5 α -R1 in rat ovary by *in situ* hybridisation

Consistent with the findings of ICC, ISH detected intense 5 α -R1 signalling that localised specifically to the theca and interstitial cells (Fig. 5.4). The signal was absent in granulosa cells.

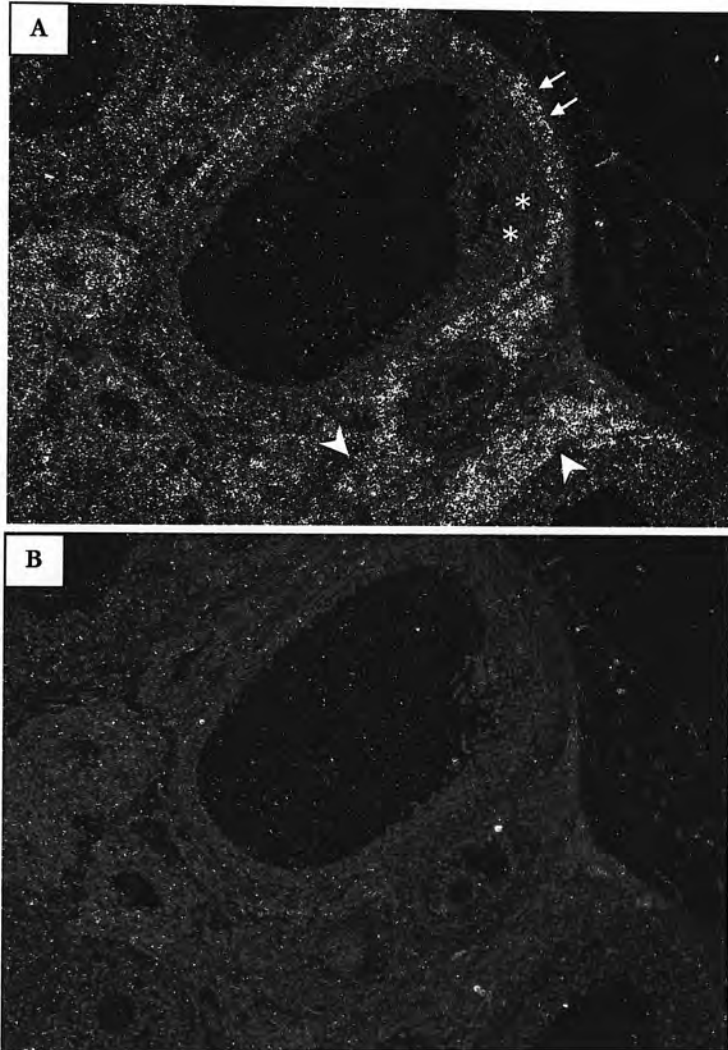


FIGURE 5.4. Localisation of 5 α -R1 mRNA by *in situ* hybridisation. *In situ* hybridisation analysis of 5 α -R1 (see "Experimental Procedures", 5.2.3) in sections of Boiuns-fixed ovaries collected from immature untreated rats which were hybridised with antisense probe (A) shows the expression of 5 α -R1 in thecal (*solid arrows*) and interstitial tissue (*arrow heads*). Note the lack of specific 5 α -R1 signal in granulosa cells (*asterisks*). A corresponding ovarian section hybridised with sense riboprobe (B) was the control for nonspecific hybridisation.

5.3.2. Effect of Gonadotrophic Stimulation on the Cellular Distribution of 5 α -R1 in Rat Ovary

The effect of gonadotrophic stimulation on the cellular distribution of 5 α -R1 protein and mRNA gene in rat ovaries stimulated *in vivo* to attain follicular maturity (Chapter 3, Experimental Procedures, 3.2.1) was investigated using ICC and ISH. Although NBF was a better fixative for ICC, to standardise tissue preparation, these studies were performed on ovaries fixed in Bouin's solution.

Figure 5.5 shows the changes in the cellular distribution of 5 α -R1 as well as the developmental changes induced in rat ovaries stimulated by eCG with or without hCG. The ovaries of intact immature and untreated rats (controls) were occupied predominantly with primary and secondary follicles, and a few small-sized antral follicles (Fig. 5.5E). Control rats had the highest levels of expression of 5 α -R1 mRNA and protein which were abundantly localised in thecal/interstitial tissue, particularly of preantral and small antral follicles (Fig. 5.5A and E). Treatment with eCG, while resulting in proliferation of the follicles and appearance of many large antral follicles, reduced markedly the expression of 5 α -R1 in thecal tissue of the enlarged antral follicles, even though the protein was still quite abundant in interstitial tissue around preantral follicles (Fig. 5.5F). However, the levels of expression of the interstitial enzyme in cells around preantral and small antral follicles was reduced significantly ($P < 0.0001$) to 80% of the control value. By ISH, 5 α -R1 mRNA expression was also reduced significantly ($P < 0.01$) by eCG to undetectable levels (Fig. 5.5B). Subsequent 12-h treatments with hCG caused further enlargement of the follicles with noticeable increments in the sizes of the thecal layers around the larger follicles (Fig. 5.5G). By inducing luteinisation, the treatment also caused the emergence of post-ovulatory follicles, of which some developed corpora lutea. In addition to the interstitial compartment where 5 α -R1 mRNA was still expressed, but at 60% of the control level (Fig. 5.5C), 5 α -R1 protein as shown by ICC was present in the thecal layers of large antral and post-ovulatory follicles to marginally detectable levels, and also occurred in the corpora lutea (Fig. 5.5G).

Notably, the immunostaining was higher in the thecal layer compared to the corpora lutea. At 24 h after hCG treatment, the ovaries had attained the largest size,

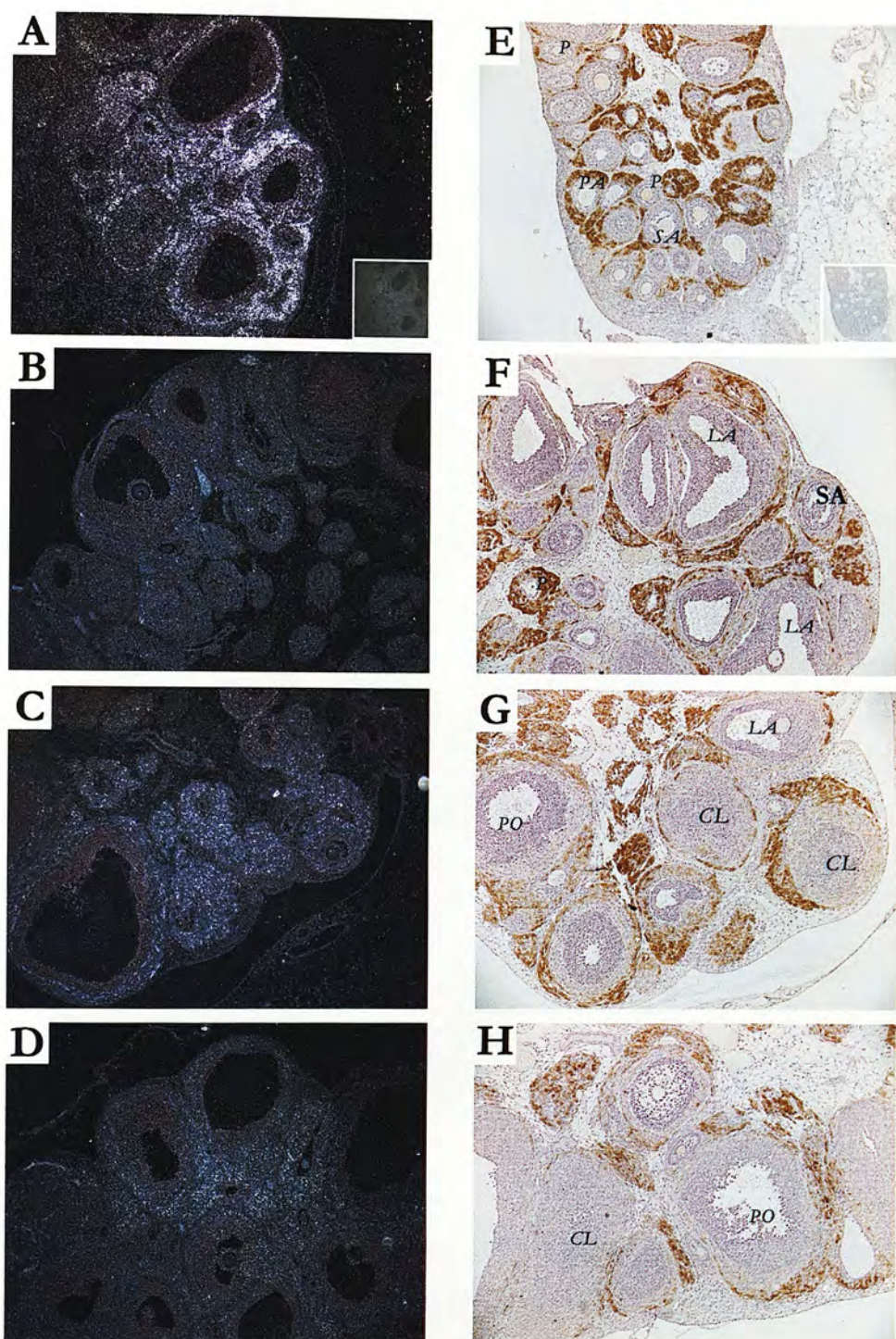


FIGURE 5.5. Gonadotrophic regulation of the cellular distribution of 5 α -R1 mRNA and protein in rat ovary. ISH (left-hand-side panels) and ICC (right-hand-side panels) analysis showed 5 α -R1 mRNA and protein, respectively, in ovaries collected from immature rats stimulated as described (Experimental procedures, 3.2.1). A & E- immature, untreated ovaries, (controls); B & F- eCG-stimulated ovaries; C & G- eCG + hCG for 12 h; D & H- eCG + hCG for 24 h. P= primary follicles; PA= preantral follicles; SA= small antral follicles; LA= large antral follicles; CL= corpus luteum; PO= postovulatory follicles.

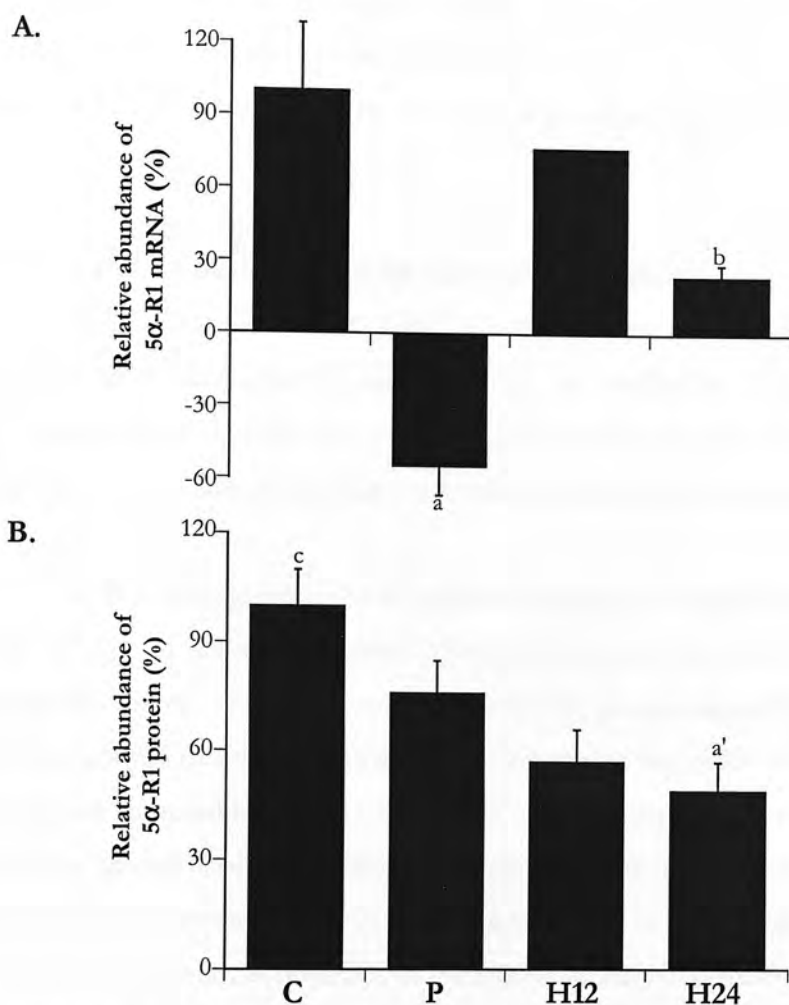


FIGURE 5.6. Gonadotrophic-controlled patterns of cellular 5α-R1 expression in the rat ovary. Using ovarian tissues from rats stimulated as described (Chapter 2, Experimental Procedures, 3.2.1), quantification of the specific 5α-R1 signals detected by ISH (A) and ICC (B) was expressed as a percentage of the control value. C= immature, untreated rats (controls); P= eCG-treated rats (48 h); H12= rats treated with eCG followed by hCG treatment for 12 h; H24= rats treated with eCG followed by hCG treatment for 24 h. Each bar represents the mean quantification (\pm SD) of specific 5α-R1 detected in 3 or 10 microscopic fields analysed on each section investigated by ISH or ICC, respectively. a, Significantly lower ($P < 0.01$) than all other treatments; b, significantly lower ($P < 0.05$) than C; a', significantly lower ($P < 0.01$) than H12; c, significantly higher ($P < 0.0001$) than all other treatments.

and were mainly occupied by post-ovulatory follicles and corpora lutea, and few large antral follicles (Fig. 5.5D and H). In comparison to the expression of 5α-R1 in the controls, the longer hCG treatment also reduced the 5α-R1 immunostaining and signal significantly ($P < 0.0001$ and $P < 0.005$) in tissues analysed by ICC and ISH, respectively. The 5α-R1 signal in interstitial tissue, although significantly reduced compared to controls, was stronger compared to thecal tissue which also had more intense immunostaining than the corpora lutea. However, the expression was still higher

compared to eCG-treated ovaries. Figure 5.6 summarises graphically the changes induced by eCG and hCG in the cellular distribution of 5 α -R1 in the rat ovary. At all stages of follicle development, the stroma and granulosa cells were essentially devoid of 5 α -R1 expression.

5.3.3. Cellular Localisation of 5 α -R1 in Adult Rat Ovary

The cellular localisation of 5 α -R1 in the adult rat ovary at different stages of the oestrous cycle, ascertained by vaginal cytology, was studied by ICC (Fig. 5.7). Attempts to localise the 5 α -R1 mRNA signal using ISH were unsuccessful because the levels of expression of the enzyme in adult ovaries were undetectable by the technique.

In the adult rat ovary, 5 α -R1 immunostaining was highest in ovaries at proestrus (Fig. 5.7A). However, the levels of expression were markedly low compared to immature ovaries. Hence, it was not possible to generate quantifiable data by Image ProPlus analysis (see Data Analysis 5.2.4). However, it was evident that 5 α -Reductase-1 was mainly localised in the interstitial tissue where its expression was marginally higher than the thecal tissue expression. Progression into the oestrus stage resulted in complete disappearance of the 5 α -R1 immunostaining in the interstitial and thecal tissue (Fig. 5.7B). Further development to the metestrus stage restored very weak expression of 5 α -R1 in the thecal tissue and also similarly resulted in weak 5 α -R1 immunostaining in the developed corpora lutea (Fig. 5.7C). By diestrus, 5 α -R1 immunostaining, although still present in the thecal and corpora lutea, was barely detectable. The granulosa and stromal tissues were clearly devoid of 5 α -R1 expression at all stages of the oestrus cycle.

5.4. DISCUSSION

Although previous reports have suggested that 5 α -R1 was present in both thecal and granulosa tissues of the rat ovary (Hillier *et al.*, 1980a; Aono *et al.*, 1981; Payne *et al.*, 1992), the immunohistochemical and *in situ* hybridisation studies described in this chapter show specifically that the cellular localisation of 5 α -R1 was restricted to the thecal/interstitial cells in the rat ovary, and was absent in ovarian stroma and granulosa cells. This distinction is probably due to the techniques used to collect the tissue investigated for 5 α -R activity. Inferring the presence of 5 α -R from studies investigating

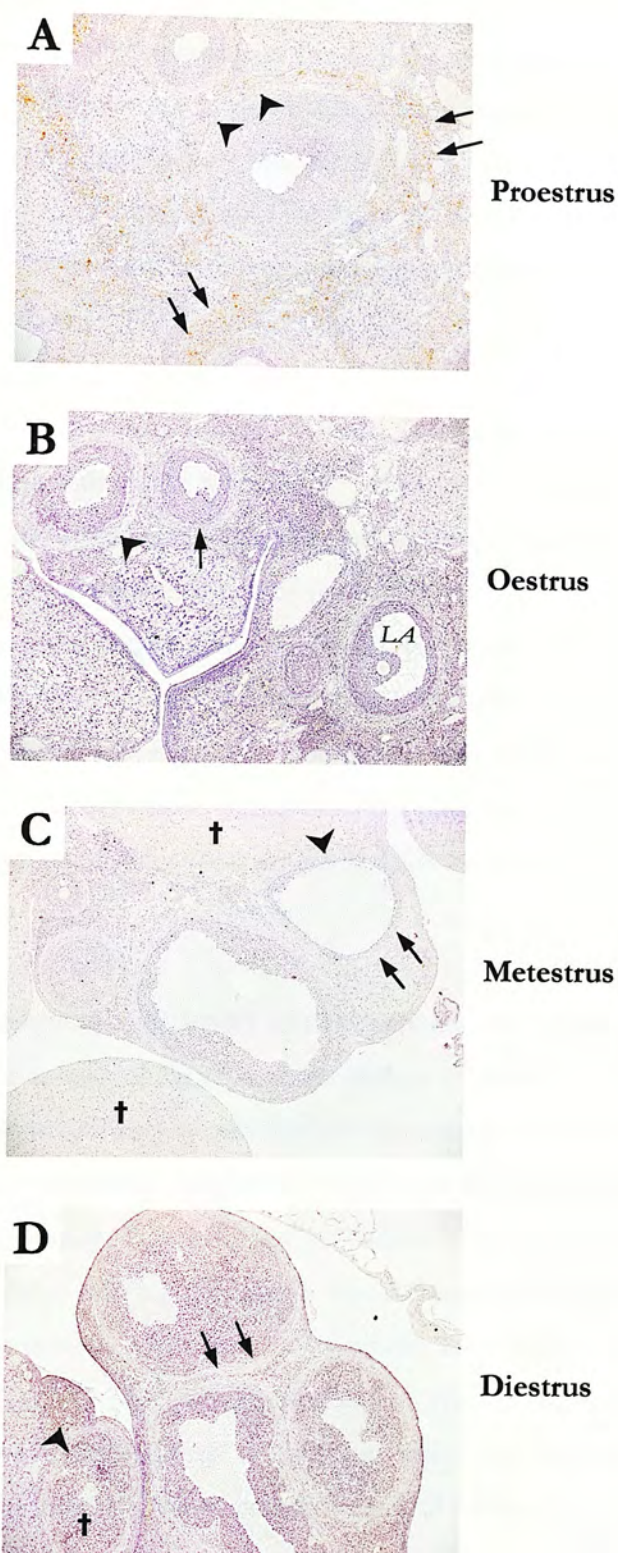


FIGURE 5.7. Cellular localisation of 5α -R1 in adult rat ovary. Ovaries collected from cyclic adult rats at different stages, (A) proestrus, (B) oestrus, (C) metestrus and (D) diestrus, of the oestrous cycle were studied by ICC of normal-buffered formalin (NBF)-fixed ovarian tissue sections (see Experimental Procedures, 5.2.2). Marked tissues are theca (*solid arrows*), interstitial tissue (*arrow heads*); granulosa cells (*asterisks*) and corpora lutea (*crosses*).

enzyme activity of granulosa cells resulted in this misconception because collection of rat granulosa cells by repeated puncture of the follicles as described in Experimental Procedures, 3.2.2 or by squeezing the ovaries between glass slides, results in contamination of the granulosa fraction with thecal/interstitial cells. It is likely that the supposed 5 α -R activity observed in such activity studies is a result of the thecal/interstitial cell contamination other than the actual localisation of 5 α -R activity in granulosa cells of rat ovary.

The distinct 5 α -R1 cellular localisation might also be species-specific since both the theca and granulosa cells of the human ovary have been shown to express 5 α -R1 mRNA and protein (Jakimiuk *et al.*, 1999; Akahira *et al.*, 2001). Although the illustration of 5 α -R2 expression was unsuccessful, it is probable that the isozyme in females is mostly expressed in non-ovarian tissues such as skin (Haning *et al.*, 1996). The abundant distribution of 5 α -R1 in liver tissue (Fig. 5.3B) while absent in heart tissue (Fig. 5.3A) is in agreement with reported tissue distribution of the enzyme (Russell & Wilson, 1994). The radial pattern of immunostaining observed in the liver tissue was similar to previous immunohistochemical studies of 5 α -reductase in rat liver (Eicheler *et al.*, 1995).

The specific expression of 5 α -R1 in the theca and interstitial cells in the rat ovary suggests that the actions of the enzyme are distinct to the cell types in which they are expressed and this is probably of relevance to the metabolism of ovarian steroids that the enzyme uses as substrates. In relation to androgen metabolism, 5 α -R1 converts androstenedione and testosterone into the 5 α -reduced steroids, 5 α -A and DHT, respectively which inhibit oestrogen synthesis by inhibiting aromatase competitively (Hillier *et al.*, 1980a). It is therefore probable that the high expression of 5 α -R1 in immature ovaries (Figs. 5.1, 5.2, 5.4, 5.5) ensures that synthesised androgens do not undergo precocious metabolism into oestrogens, which are required for normal physiology in adult, postpubertal female animals. It is well known that in adults, normal follicle growth that culminates in ovulation and hence, ensures fertility, requires FSH-induced developmental changes that equip developing follicles with structural and steroidogenic maturity which is dependent on oestrogens (Hillier *et al.*, 1988; Palter *et al.*, 2001; Rosenfeld *et al.*, 2001). The changes in the localisation of 5 α -R1 in hormonally

stimulated ovaries in immature rats (Fig. 5.5) or adult cyclic ovaries (Fig. 5.7) illustrate the regulatory role of gonadotrophins in the enzyme's cellular distribution in the rat ovary.

The stimulation of ovaries of immature rats *in vivo* allowed the study of the effects of gonadotrophic regulation of 5 α -R1 in an animal model. Whereas the enzyme was highly expressed in the thecal/interstitial cells in ovaries of immature rats (Fig. 5.5A & E), the down-regulation of its expression in thecal cells of larger antral follicles by eCG (Fig. 5.5B & F) suggests that the enzyme is downregulated by FSH. In fact, this FSH effect was clearly demonstrated by *in situ* hybridisation where the expression of 5 α -R1 mRNA in both theca and interstitial cells was reduced significantly ($P < 0.01$) by eCG (Fig. 5.5B). The findings agree with other studies that reported the inhibitory effect of FSH on 5 α -R1 (Aono *et al.*, 1981; Payne *et al.*, 1992). Whereas the 5 α -R1 mRNA gene was completely turned off by eCG (Fig. 5.5B), the protein was still present in the interstitial cells of preantral and small antral follicles (Fig. 5.5F) suggesting a temporal association between the gene expression and protein synthesis. The abundant expression of 5 α -R1 protein in interstitial cells of eCG-stimulated ovaries, albeit reduced compared to controls, while almost absent in thecal cells might be due to the expression of the protein lagging behind the gene expression. When eCG switched off the 5 α -R1 gene, similar effects on the protein expression would be expected to follow, as suggested in studies of adult rat ovaries where the decline in 5 α -R1 protein activity lagged hours behind the gene expression (Lephart *et al.*, 1992). The restoration of detectable 5 α -R1 immunostaining within thecal cells by hCG administered for 12 h (Fig. 5.5G) showed the stimulatory effect of LH on 5 α -R1 expression, as previously reported (Terakawa *et al.*, 1978; Fukuda *et al.*, 1979; Aono *et al.*, 1981). However, the increase due to the 12-h hCG-treatment was transient, as shown by the significantly lower expressions of 5 α -R1 mRNA in ovaries treated for 24 h ($P < 0.05$).

Although present in adult cyclic ovaries as shown by immunohistochemistry (Fig. 5.7), the expression of 5 α -R1 was very low and could not be demonstrated by *in situ* hybridisation. It is probable that the enzyme does not have as prominent a role in adult ovarian function as in the immature ovary. However, the 5 α -R1 expression was developmentally regulated throughout the oestrous cycle. The highest levels of 5 α -R1

expression occurred during proestrus, and the enzyme was localised in the interstitial tissue. The thecal cells of the adult ovary were essentially devoid of 5 α -R1 protein suggesting that 5 α -R1 activity is lost from thecal tissue, but retained at very low levels in interstitial tissue. It is unclear why this change in cellular distribution occurs. Possibly, the removal of the inhibitory effect of 5 α -reduced steroids, particularly androgens, is the driving requirement behind the loss of 5 α -R1 expression in thecal tissue of adult ovaries. The physiological consequence is the removal of aromatase inhibitors, which reduce aromatase activity that is crucial for reproductive function. Examples of aromatase 'action' are the oestrogenic effects in the ovary and vaginal wall. Oestrogens induce concomitant proliferation of the epithelial cells of the vagina in readiness for coitus (Johnson & Everitt, 1995), and ovarian granulosa cells in preparation of oocyte release at ovulation (Gaytan *et al.*, 1996; McGee & Hsueh, 2000). The effect on rodent vaginal cytology is particularly marked such that the stages of the oestrous cycle can be assessed quite accurately by examination of the cell types present in smears of swabs of the vaginal epithelium. The ovarian and vaginal changes are vital for successful reproduction, and any hindrance to oestrogen release, particularly, oestradiol, the most potent oestrogen, must be removed to ensure fertility. Therefore, the changes in the cellular distribution of 5 α -R1 protein in the adult ovary during the stages of the oestrous cycle are likely to be a response to this requirement.

During proestrus, rising FSH levels induce granulosa cells to produce rising levels of oestrogens, which drive different reproductive processes. As a result, oestrus, the period when FSH is at peak level, is marked with complete loss of 5 α -R1 expression, even in the thecal cells (Fig. 5.7B). The critical dependence of oestrus on oestrogen production requires removal of the ovarian mechanisms that inhibit aromatase activity. Following the drop of FSH as the cycle develops, production of oestrogens by the granulosa cells also drops precipitously such that the proliferative effects on the vaginal epithelium are removed. During this metestrus phase, the rising levels of LH which induce synthesis of progesterone also stimulate 5 α -R1 expression which is then expressed faintly in the interstitial and the corpora lutea that develop (Fig. 5.7C). Since progesterone is readily metabolised by 5 α -R1 and is a preferred substrate for the enzyme than androgens (Eckstein & Nimrod, 1977; Normington & Russell, 1992; Payne *et al.*, 1992), 5 α -R1 action probably converts progesterone into 5 α -reduced

pregnanes which have been suggested to improve reproductive function in oestrogen-primed rats by facilitating the females' sexual receptivity of the males (Frye & Vongher, 2001). By diestrus, which is marked by low oestrogen:progesterone ratios, the expression of 5 α -R1 is reduced in both interstitial tissue and corpora lutea (Fig 5.7D).

Studies in 5 α -R1 knockout mice have shown that the enzyme is crucial for successful pregnancy outcome through its conversion of progesterone into 5 α -reduced pregnanes which are necessary for cervical ripening (Mahendroo *et al.*, 1999) suggesting that in the adult, 5 α -R1 is a more important enzyme in the endocrinology of pregnancy rather than preovulatory follicular development. Infact, the uterus was shown to be a site for 5 α -R action where progesterone was metabolised, particularly in oestrogen-primed animals into various steroids among which were 5 α -reduced pregnanes (Verma & Laumas, 1976). Therefore, it is likely that 5 α -R1 in the adult female has a more important role in uterine rather than ovarian endocrinology.

Interestingly, in women, increased activity of 5 α -R1 is indicated in polycystic ovarian disease (Agarwal *et al.*, 1996; Jakimiuk *et al.*, 1999). Evidence for this was investigated by measurement of 5 α -reduced androgens in polycystic ovaries, which is discussed in Chapter 6.

Chapter 6. Measurement of 17-ketoandrogens (17KA), androstenedione and oestradiol levels in normal and PCOS follicular fluids

6.1 INTRODUCTION

Since the description of polycystic ovary syndrome (PCOS) as a major infertility problem arising from anovulation (Stein & Leventhal, 1935), the syndrome has been well recognised as a common endocrinopathy of women of reproductive age (Franks & White, 1993; Knochenhauer *et al.*, 1998; Franks *et al.*, 1999; Marshall, 2001). However, there are many causes of anovulation, and hence, of PCOS. While the pathogenesis of PCOS remains elusive and is still a subject of controversial debate, a common symptom is hyperandrogenemia (Barnes, 1997; Legro *et al.*, 1998; Nelson *et al.*, 1999; Dunaif & Thomas, 2001; Zborowski *et al.*, 2001). Whether secreted by either or both androgen-secreting organs, the ovaries or adrenals, the circulating high levels of androgens contribute significantly to the clinical presentation of PCOS (Speroff *et al.*, 1983). Of particular interest in this study, are the 5 α -reduced androgens that are part of the hyperandrogenemia of PCOS (Lawrence, 1968; Laatikainen *et al.*, 1980; Matteri *et al.*, 1989; Agarwal *et al.*, 1996; Franks *et al.*, 1999; Jakimiuk *et al.*, 1999).

Although the involvement of 5 α -reduced androgens in PCOS is still unclear, their inhibitory effect on aromatase action *in vitro* (Hillier *et al.*, 1980) suggests a modulatory role of ovarian follicle development via their effects on oestrogen production. Since the levels of steroids are much higher in the ovary as compared to the general circulation, it is likely that 5 α -reduced androgens inhibit follicle development locally. Therefore, measurement of the levels of 5 α -A, the 5 α -reduced metabolite of androstenedione, the principal androgenic substrate in human ovaries, as well as other 5 α -reduced androgens in follicular fluid, is important in assessing the magnitude of the role of 5 α -reduced androgens in the aetiology of PCOS. In the absence of a standard method to measure 5 α -A, a RIA was developed to quantify 5 α -A. The availability of an antibody that was raised against androsterone, but has high cross-reactivity with 5 α -A and androstenedione, enabled the development of a RIA, which measured 5 α -A, androsterone and androstenedione simultaneously, hereafter defined as 17-keto androgens (17KA) RIA. As described by Hillier (1974), androsterone, a small non-

immunogenic molecule (MW= 290.4) was linked covalently to bovine serum albumin (BSA), a carrier molecule, to form androsterone-3-BSA, which is immunogenic. Injection of androsterone-3-BSA into rabbits raised antibodies against androsterone-3-BSA. Characterisation of the rabbit serum containing the antibodies (antiserum) showed that the antiserum cross-reacted and had greater affinity for 5 α -A than the parent steroid, a property used to establish a RIA for 17KAs. Thus by performing this assay on biological samples along with separate specific RIAs of androstenedione and oestradiol, it was possible to estimate relative concentrations of 5 α -reduced-17-ketoandrogens (5 α 17KAs), androstenedione, and oestradiol in follicular fluids of normal and PCOS subjects.

6.2. EXPERIMENTAL PROCEDURES

6.2.1. Titration of Anti-Androsterone Serum

Rabbit anti-androsterone-3-BSA serum (provided by Prof. S.G. Hillier, Univ. of Edinburgh, Dept. of Reproduction and Developmental Sciences, Little France, UK) was stored at -20°C and diluted (1:100) as required in phosphate buffered saline containing 0.1% gelatine, pH 7.2 (PBS) to obtain a stock solution, which was also stored as 1.0 ml aliquots at -20°C.

After an initial dilution (1:2.5) of stock antiserum in PBS, 200 μ l of serial dilutions (1:4) in PBS were mixed with a constant amount of [3 H]androsterone (\sim 10,000 cpm/200 μ l PBS). The reactions were incubated overnight at 4°C. To terminate incubation, 0.5ml 1% dextran charcoal suspension was added to the reactions, which were vortexed and incubated on ice for 10 min to separate bound and free [3 H]androsterone. After centrifugation at 2500 g for 10 min at 4°C, the supernatant (containing free [3 H]androsterone) was decanted into vials to which 3.5 ml scintillation fluid was added. The radioactivity was then measured by β -scintillation counting (Model Tri-Carb 2100TR, Packard, A Canberra Company, USA). Plots of the radioactivity against the antiserum dilutions generated titration curves from which the working antiserum dilution (titre) was obtained.

6.2.2. Preparation of 17KA RIA standard curve

200 µl aliquots of freshly diluted antiserum (1:2000 in PBS) and 200 µl [³H]androsterone (~10,000 cpm diluted in PBS) were added to duplicate tubes containing 100 µl of 5α-A standard solutions of concentrations, 0.04, 0.08, 0.16, 0.31, 0.625, 1.25, 2.5, 5.0, 10.0 and 20.0 ng/100 µl. The standard solutions were prepared from the 5α-A stock solutions of concentrations of 20 ng/100µl by serial dilutions in PBS. After vortexing, the reactions were incubated overnight at 4°C. Separation of the bound and free [³H]androsterone by dextran charcoal (as described above) yielded fractions of unbound radiolabelled steroid that were inversely proportional to the amount of added unlabelled 5α-A. The plots of the specific binding against the 5α-A standard concentrations (standard curves) used to measure the steroid concentrations were calculated by an assay calculator (Assay Zap, Universal Assay Calculator, Biosoft, Cambridge, UK).

6.2.3. Specificity of 17KA RIA

The relative affinities of the heterologous steroids, androsterone, DHT, androstenedione, testosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, oestradiol and progesterone, for the androsterone antiserum were determined by competitive binding with 5α-A. Stock solutions of the listed steroids (pure grade) in ethanol (2mg/ml) were diluted 1000-fold in ethanol followed by a 10-fold dilution in PBS to form standard solutions at a concentration of 20ng/100µl. Serial dilutions of these stock solutions in PBS generated steroid solutions with concentrations ranging 0.04 - 20 ng/100µl, which were assayed alongside the 5α17KA standard curve described above. Taking arbitrarily the crossreaction of 5αA in this system as 100%, the percent cross-reaction of steroid X is calculated at 50% displacement of [³H]androsterone. The percent cross-reaction of X equals:

$$\frac{\text{mass of 5}\alpha\text{A required to displace 50\% of } [^3\text{H}]\text{androsterone}}{\text{mass of steroid X required to displace 50\% of } [^3\text{H}]\text{androsterone}}$$

6.2.4. Synthesis of [³H] 5α-Androstenedione:

6.2.4.1. By COS cells transfected with 5α-R1 cDNA

Simian COS-7 cells were transfected with 5α-R1 cDNA as described (Qiagen Superfect, UK). 5α-Reductase-1 activity was then used to synthesise [³H]5α-A *in vitro* as

described (Andersson & Russell, 1990) except that the radiolabelled steroid, incubation periods and TLC system used were [^3H]androstenedione (final specific activity- 93 $\mu\text{Ci}/\text{mmol}$), 30 and 60 min, and alumina pre-coated TLC sheets developed with cyclohexane:toluene: ethanol (100:95:5 v/v/v; see Chapter 3, 3.2.8.1.), respectively. The conversion of [^3H]androstenedione to [^3H]5 α -A was monitored by radiochromatography.

6.2.4.2. By endogenous 17 β -HSD2 in COS cells

To exploit the 17 β -HSD activity endogenous in COS cells (Poutanen *et al.*, 1993), non-transfected Simian COS-7 cells were treated as described above except for the radiolabelled steroid and co-factor which were changed to [^3H]DHT (final specific activity 100 $\mu\text{Ci}/\text{mmol}$), and 1 mM NAD, respectively.

6.2.4.3. Purification of [^3H]5 α -A

Tritiated 5 α -A was purified by TLC (Chapter 3, 3.2.8.1). Recovery of [^3H]5 α -A from the TLC sheets was achieved by solvent elution using the apparatus shown in Fig. 6.1. The position of separated [^3H]5 α -A on the TLC sheets was identified by running 5 α -A standards (2 μg) on the sides of the sheets at positions widely spaced from the experimental samples to avoid contamination. The positions in the lanes containing the synthesised [^3H]5 α -A were excised and eluted with 4 ml methanol. The eluate was collected in 16 x 100 mm borosilicate glass tubes (Corning Inc. NY, USA). To ascertain the radioactivity and purity of the synthesised [^3H]5 α -A, 50 μl aliquots of the eluate were analysed by scintillation counting as described in section 6.2.2, and also dried under nitrogen gas in a heated solid block at 55°C for analysis by radiochromatography (Chapter 3, 3.2.8.1). The remaining fraction of the eluate was dried similarly and the dry residues of purified radiolabelled [^3H]5 α -A were dissolved in 1 ml toluene: ethanol (9:1 v/v). The 30- and 60-min [^3H]5 α -A preparations were pooled and stored at -20°C until further use.

6.2.5. Extraction of Steroids from Follicular Fluid Samples

Follicular fluids were diluted 1 in 10 PBS. Duplicate 100 μl aliquots of the dilutions were transferred into 16 x 100 mm glass tubes. To each sample, a solution of [^3H]5 α -A in ethanol (~ 2000 dpm) was added as an internal recovery standard. Identical

aliquots were transferred to scintillation vials in duplicate for determination of the total radioactivity added. Extraction of the steroids was performed with 1 ml diethyl ether as described (Chapter 3, 3.2.7) and followed by dissolving in 500 μ l PBS, which was completed by incubation in a waterbath at 37°C. After vortexing, 250 μ l of the PBS solution was transferred into a scintillation vial for counting to assess the recovery of [3 H]5 α -A added to the follicular fluid samples. Duplicate 100 μ l aliquots of the remaining PBS solution were analysed by the 17KA RIA. In addition, duplicate 100 μ l aliquots of the 1 in 10 dilution in PBS were measured for androstenedione and oestradiol by RIA.

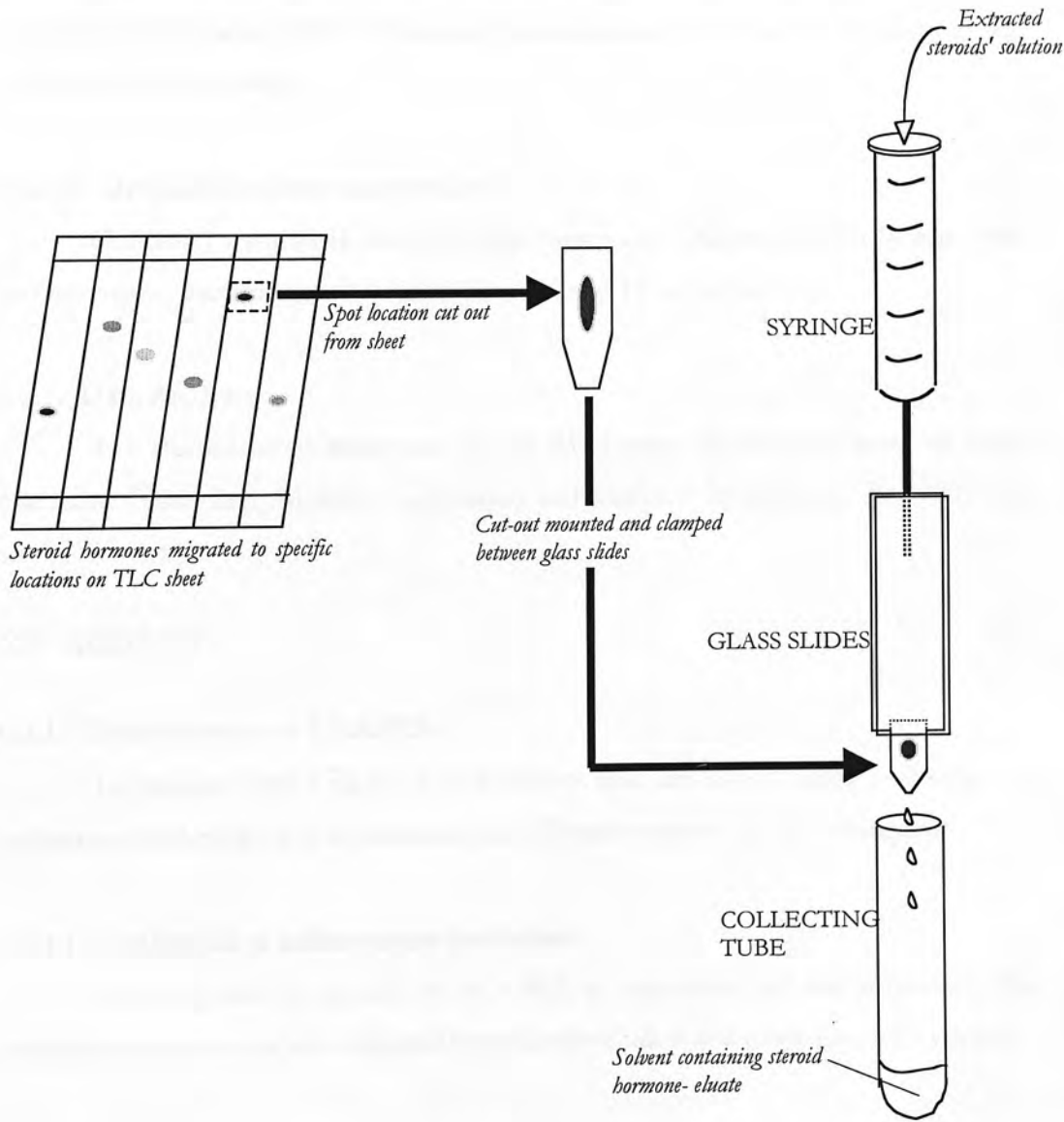


FIGURE 6.1. Elution apparatus for recovery of steroids after TLC

6.2.6. Radioimmunoassays

6.2.6.1. 17KA radioimmunoassay

5 α -androstenedione, androsterone and androstenedione were measured in a single assay as described (Section 6.2.3) using an antibody that showed a high degree of crossreactivity with all three steroids. The inter-assay coefficients of variation for the "low" (0.4 ± 0.1 ng/100 μ l) and "high" (2.4 ± 0.3 ng/ 100 μ l) 5 α -A quality control solutions were 22% and 14%, respectively.

6.2.6.2. Androstenedione radioimmunoassay

Androstenedione was measured in extracted follicular fluid samples as described previously (Hillier *et al.*, 1991). The intra- and inter-assay coefficients of variation were 11% and 9%, respectively.

6.2.6.3. Oestradiol radioimmunoassay

Oestradiol was measured as described previously (Chapter 3, 3.2.8.2). The intra- and inter-assay coefficients of variation were 8% and 11%, respectively.

6.2.7. Data Analysis

The steroid levels measured by the RIAs were determined using an assay calculator (AssayZap, Biosoft, Cambridge) and analysed by one-way ANOVA and student's t-test.

6.3. RESULTS

6.3.1. Establishment of 17KA RIA

To measure the 17KAs, a RIA system was developed using androsterone antiserum, unlabelled 5 α -A as standards and [3 H]androsterone as the radioligand.

6.3.1.1. Evaluation of androsterone antiserum

Knowing that the specificity of a RIA is dependent on the antiserum, the androsterone antiserum was evaluated by antibody dilution and cross-reactivity studies.

I. Determination of antiserum titre

The dilution of the androsterone antiserum used to set up the 17KA RIA, 1:2000 (Fig. 6.2), corresponded to 50% binding of [^3H]androsterone (see "Experimental Procedures, 6.2.1").

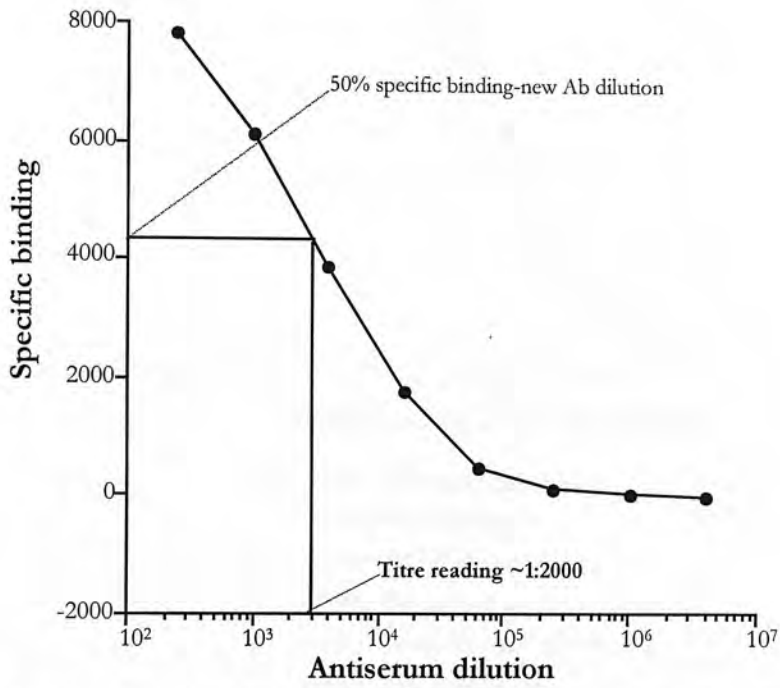


FIGURE 6.2. Antibody dilution curve used to define antiserum titre for the 17KA RIA. The specific binding of [^3H]androsterone in serial dilutions (1:4) of stock antiserum solution (see "Experimental Procedures, 6.2.1") was employed to identify the antiserum titre that was used to establish a 5 α -A RIA. The titre read off the plot of specific binding versus dilution of antiserum (antibody dilution curve), corresponding to 50% specific binding of the antiserum, 1:2000, was used in the 17KA RIAs.

II. Specificity of anti-androsterone serum

The relative specificity of the antiserum for 5 α -A was determined by comparing the relative affinity of the antiserum for various steroids structurally similar to 5 α -A and known to be present in follicular fluid as described (Experimental Procedures, 6.2.3).

Androsterone and androstenedione cross-reacted significantly with the antiserum (Fig. 6.3). On the other hand, cross-reaction with testosterone, DHT, 5 α -androstane-3 α ,17 β -diol, 5 α -androstane-3 β ,17 β -diol, oestradiol and progesterone was minimal (< 0.1%). Therefore, the assay showed class specificity for 17ketoandrogens. Table 6.1 lists the cross-reactions of the steroids with the antiserum.

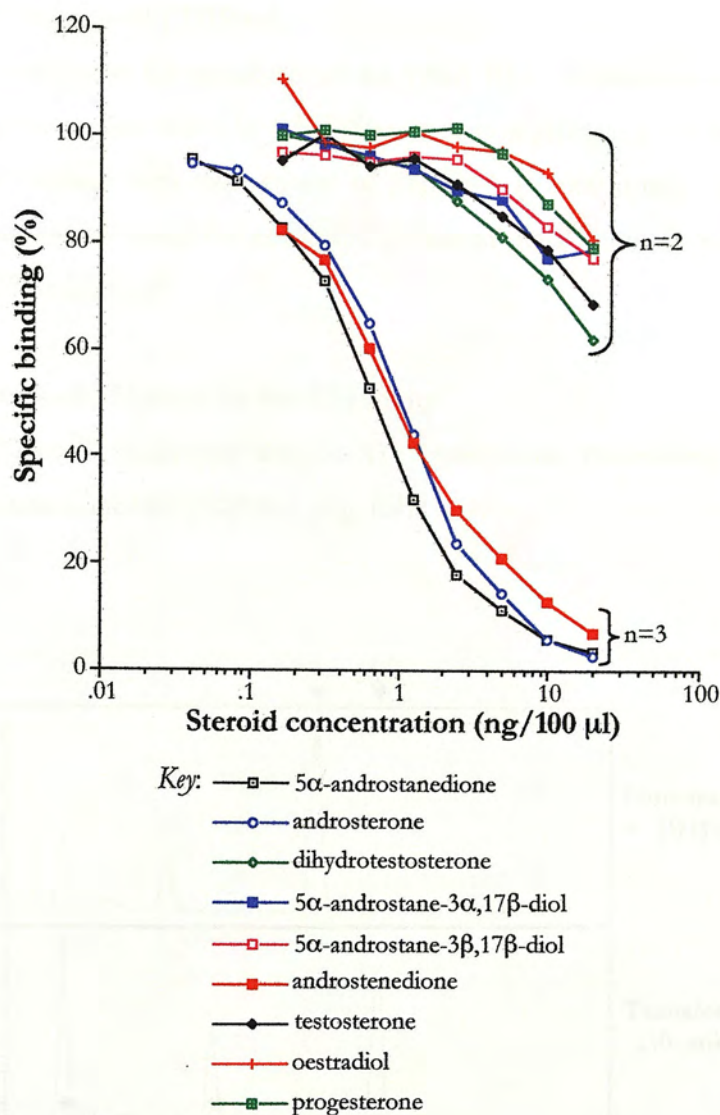


FIGURE 6.3. Cross-reaction of heterologous steroids with anti-androsterone serum used for the 17KA RIA.

Steroid	Cross-reaction* (%)
5α-androstenedione	100
androstenedione	70
androsterone	63.6
dihydrotestosterone	< 0.1
testosterone	< 0.1
5α-androstane-3α,17β-diol	< 0.1
5α-androstane-3β,17β-diol	< 0.1
oestradiol	< 0.1
progesterone	< 0.1

TABLE 6.1. Cross-reactions of various steroids with anti-androsterone antibody used for the 17KA RIA.

*Calculated as: $\frac{\text{ng 5}\alpha\text{-A at 50\% binding}}{\text{ng heterologous steroid at 50\% binding}} \times 100$

6.3.1.2. Synthesis of [^3H]5 α -A

To improve the specificity of the 17KA RIA, the steroids were extracted with ether prior to measurement by RIA. The extraction efficiency was assessed by analysis of samples spiked with trace counts of [^3H]5 α -A (~2000 dpm). Tritiated 5 α -A was synthesised *in vitro* using the enzyme activities of 5 α -R1 transfected and endogenous 17 β -HSD2 in COS cells.

I. Synthesis of [^3H]5 α -A by 5 α -R1 activity

COS cells transfected with 5 α -R1 (Experimental Procedures, 6.2.4.1) converted [^3H]androstenedione into [^3H]5 α -A (Fig. 6.4).

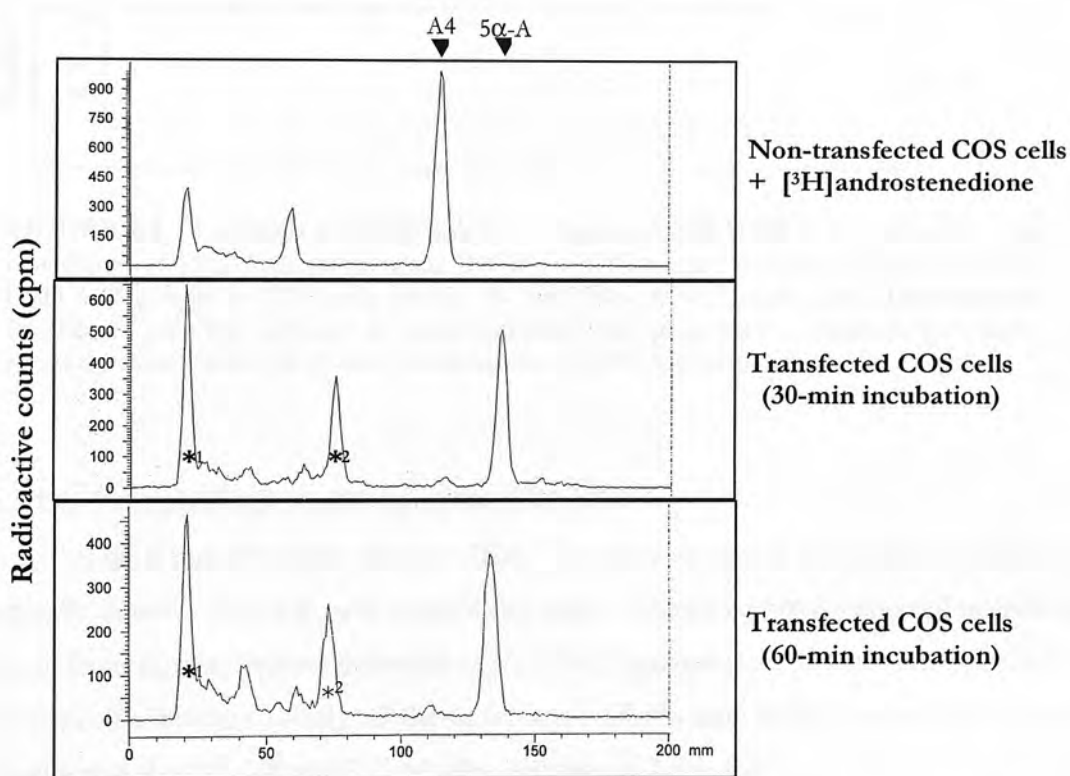


FIGURE 6.4. Synthesis of [^3H]5 α -A by COS-7 cell transfected with 5 α -R1. The conversion of [^3H]androstenedione (A4) to [^3H]5 α -androstenedione (5 α -A) by COS cells transfected with 5 α -R1 (Experimental Procedures, 6.2.4.1) is shown by radiochromatography performed on alumina pre-coated sheets developed with cyclohexane:toluene:ethanol (100:95:5, v/v/v) solvent system. *1: unknown non-polar steroid metabolite; *2: unknown polar steroid metabolite.

II. Synthesis of [³H]5α-A by 17β-HSD activity

Endogenous 17β-HSD activity in COS cells (Poutanen *et al.*, 1993) also converted [³H]DHT to [³H]5α-A as shown in Fig. 6.5.

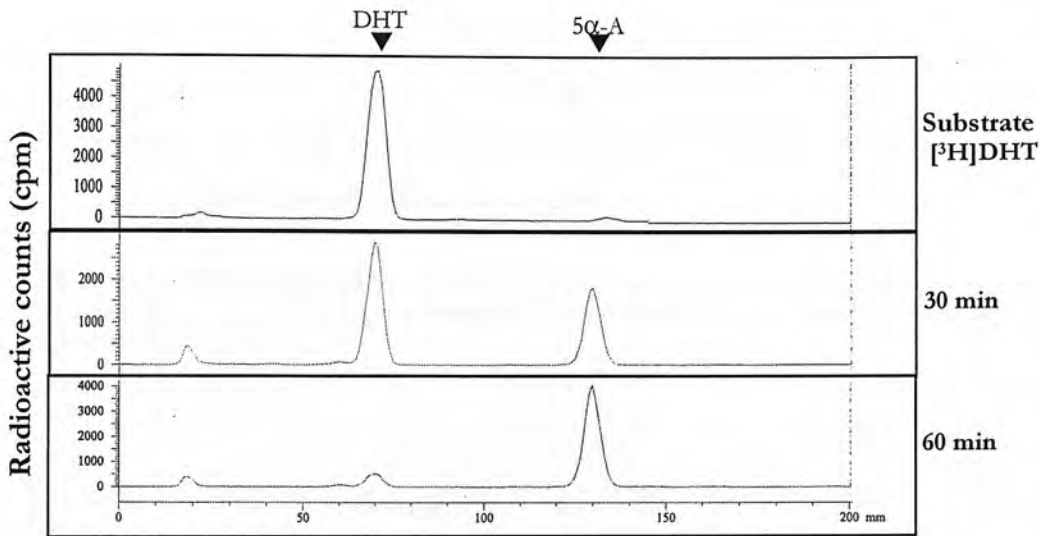


FIGURE 6.5. Synthesis of [³H]5α-A by endogenous 17β-HSD in COS-7 cells. The conversion of [³H]dihydrotestosterone (DHT) to [³H]5α-androstanedione (5α-A) by 17β-HSD endogenous in COS cells during 30- and 60-min incubations (see "Experimental Procedures", 6.2.1.2) is shown by radiochromatography performed on alumina pre-coated sheets developed with cyclohexane:toluene:ethanol (100:95:5, v/v/v).

6.3.1.4. Precision and sensitivity of 5α-17KA RIA

The standard curves of the 17KA RIA (Experimental Procedures, 6.2.3) were typically sigmoid (Fig. 6.6), and covered the range of levels of 5α-A expected in follicular fluids from normal ovaries (Milewich *et al.*, 1992; Agarwal *et al.*, 1996). The specific and non-specific binding (NSB) of the assay were 53.9% and 3.7%, respectively (n=20). Precision at the 20th, 50th and 80th quartiles is shown in Table 6.2.

The sensitivity of the assay was 0.02 ng/100 μl (0.67 nM). The intra- and inter assay variability, determined using pooled follicular fluids with 5α-A levels of 2.62 ± 0.07 ng/ 100 μl (mean ± SD, n= 4), were 10.4% and 10.6%, respectively.

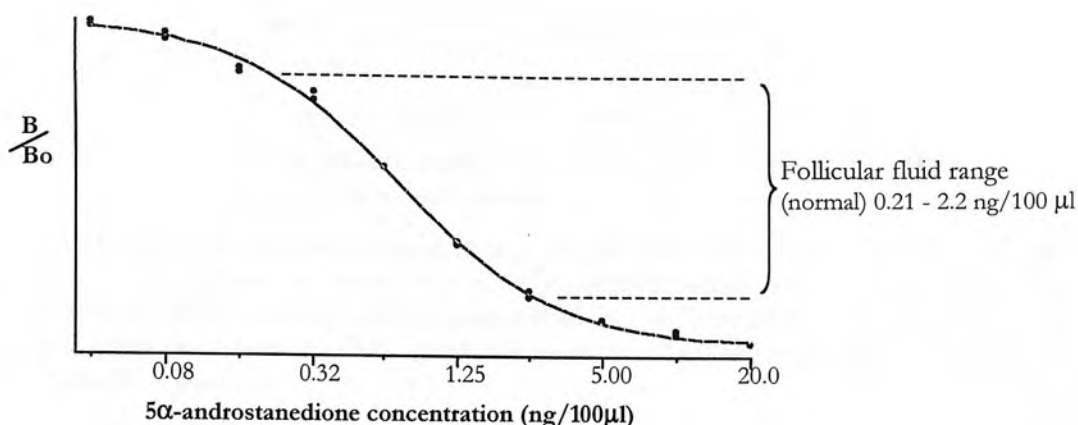


FIGURE 6.3. Standard curve of 17KA RIA. The sigmoid standard curve prepared with 0.04 - 20.0 ng/100 μ l range of 5 α -A concentrations was generated by Assay Zap Universal Calculator (Biosoft, Cambridge, UK).

Quartile	Mean \pm SD (n=20)
ED 20	2.7 \pm 0.9
ED 50	0.8 \pm 0.3
ED 80	0.2 \pm 0.1

TABLE 6.2. The precision of the standard curve of the 17KA RIA was shown by the 20, 50 and 80 effective doses.

6.3.1.4. Determination of accuracy of 17KA RIA

To determine the accuracy of the RIA for 5 α -A, the concentration of 5 α -A in pooled follicular fluid samples with and without 10 ng 5 α -A added, was measured by the RIA (Table 6.3). Adjusting for extraction and buffering losses, the differences in the concentrations of 5 α -A between follicular fluid samples where 5 α -A steroid was added and those without added steroid were on average, 12.2 ± 1.1 ng/100 μ l, values that correlate well with the amount added, i.e., 10 ng/ 100 μ l. The extraction and recovery losses were assessed by recovery of [3 H]5 α -A added to the samples and were determined as percentages of the added radioactivity. Blanks obtained by extracting PBS and distilled water (in parallel to the test samples) were undetectable.

Sample	5 α -A (ng/ 100 μ l)
FF pool	2.2 \pm 0.3
FF pool + 10 ng 5 α -A	14.4 \pm 1.1
10 ng 5 α -A (in buffer)	9.2 \pm 0.8

TABLE 6.3. The concentrations of 5 α -A in samples of pooled follicular fluid (FF) with and without added 5 α -A at known concentration were measured after ether extraction (see "Experimental Procedures, 6.2.5) in quadruplicate in 3 replicate RIAs. The values are 5 α -A concentrations (mean \pm SEM) measured in each indicated group after adjusting for extraction losses.

6.3.2. Measurement of 17KAs in follicular fluid of normal and PCOS women.

Although the two TLC systems developed , i.e., alumina- and silica pre-coated sheets, could separate the follicular steroids, specifically androsterone and androstenedione from 5 α -A as shown, (Chapter 3, Figs. 3.4 and 3.8), they were problematic in the complete recovery of [3 H]5 α -A. As a result, the assays on follicular fluid were done without the chromatographic step, and hence, measured the two 5 α -17ketoandrogens, 5 α -A and androsterone, and androstenedione simultaneously. Follicular fluids from normal and polycystic ovaries were provided by Prof. S. Franks (Department of Reproductive Science and Medicine, Imperial College School of Medicine, London, UK). The follicular fluids analysed are described in Table 6.4.

	Normal	Ovulatory PCOS	Anovulatory PCOS
Phase of menstrual cycle	Follicular	Follicular	-
Number of samples	39	44	36
Follicle size:			
Mean	13.9 mm	8.4 mm	6.3 mm
Minimum	5 mm	4 mm	4 mm
Maximum	27 mm	21 mm	9 mm
Range	22	17	5

TABLE 6.4 Description of the follicular fluids.

The results of the RIAs of the follicular fluids are summarised in Table 6.5 and Fig. 6.7.

RIAs	Normal	Ovulatory PCOS	Anovulatory PCOS
17KA	364.5 \pm 46.3	828.7 \pm 168.1	618.9 \pm 127.5
androstenedione	143.8 \pm 15.2	264.3 \pm 34.1	183.8 \pm 26.9
oestradiol	2465.3 \pm 618.6	775.7 \pm 367.0	342.8 \pm 80.2

Table 6.5. The concentrations of 17KAs, androstenedione and oestradiol in follicular fluids from ovaries of normally menstruating (normal) and polycystic ovulating (ovulatory PCOS) women in the follicular phase of the menstrual cycle, and women with polycystic anovulation (anovulatory PCOS) were measured in duplicate using the RIAs described in “Experimental Procedures, 6.2.6.” The values are mean \pm SEM steroid concentrations (nM) measured in each group.

The levels of the 17KAs were elevated significantly in follicular fluids from women with both ovulatory ($P < 0.01$) and anovulatory ($P < 0.05$) PCOS compared to follicular fluids of normal women (Fig. 6.7A). By comparison, the levels of androstenedione were only raised significantly in ovulatory PCOS women when compared with follicular fluids of normal women ($P < 0.01$; Fig. 6.7B). This increase was also significant when compared to anovulatory follicular fluids ($P < 0.05$). In contrast, comparisons of the levels of oestradiol in the same follicular fluids showed that follicular fluids from normal women were significantly higher compared to the PCOS subjects, whether ovulatory ($P < 0.05$) or anovulatory ($P < 0.01$; Fig. 6.7C).

6.4. DISCUSSION

Previous studies of the metabolism of 5α -A have shown that it is synthesised in the human ovary (McNatty *et al.*, 1979; Agarwal *et al.*, 1996) through the action of 5α -R1, which is expressed locally (Haning *et al.*, 1996). Of interest in this study, 5α -A was previously implicated to be a major factor in the endocrinopathy of PCOS (Jakimiuk *et al.*, 1999; Agarwal *et al.*, 1996). However, the absence of a standardised method for the measurement of the steroid created limitations in the quantification of the steroid. In addition, an antiserum that is specific to 5α -A is still unavailable because 5α -A is structurally similar to other 17keto steroids, particularly androsterone and androstenedione (Chapter 1, Fig. 1.7). The high degree of crossreaction of antisera with both androstenedione and androsterone is a problem that has been reported in other studies (Milewich *et al.*, 1992; Agarwal *et al.*, 1996). As a result, during the development of the methods used to measure 5α -A in follicular fluid

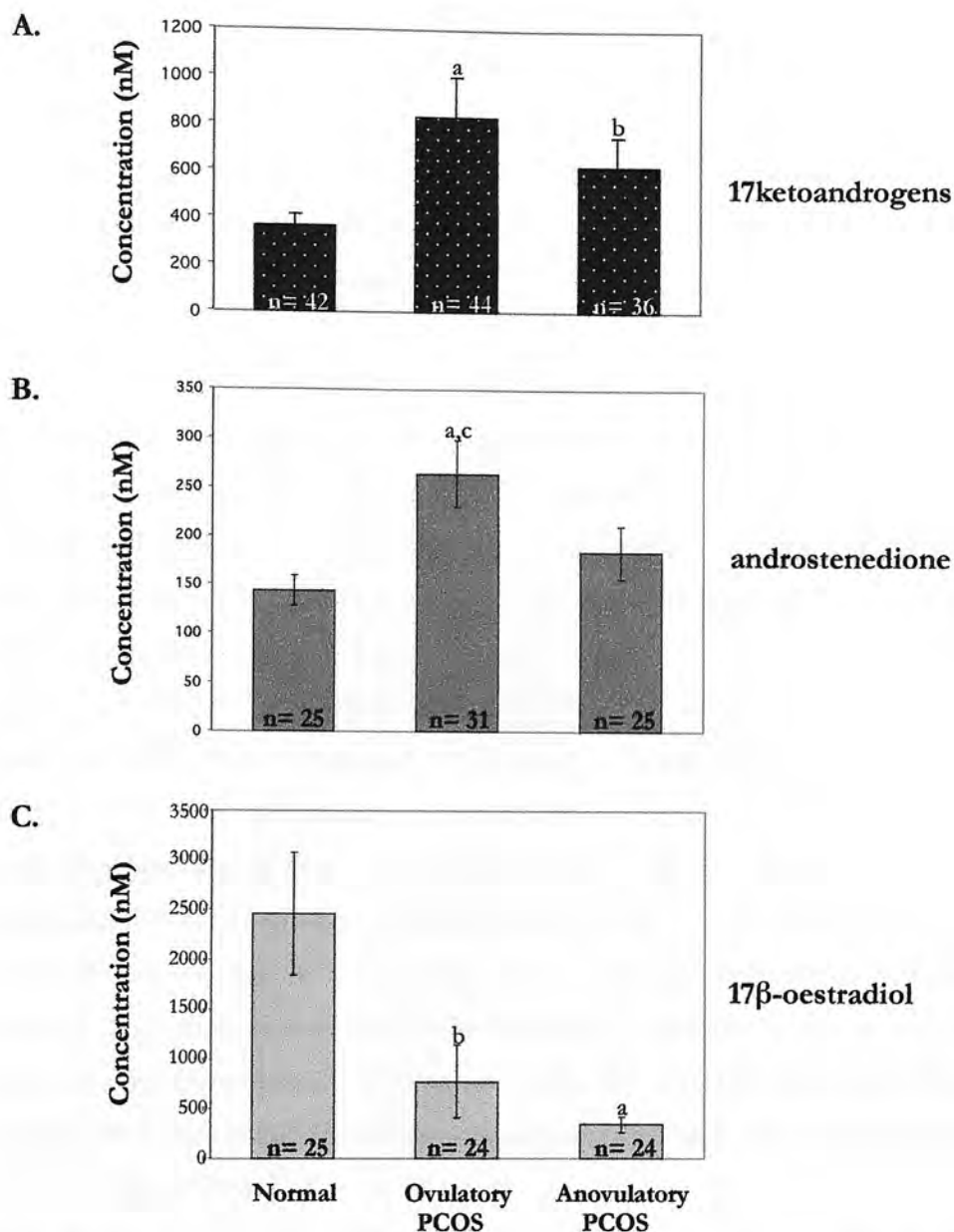


FIGURE 6.7. Comparison of 17ketoandrogens, androstenedione and 17β-oestradiol measured in follicular fluids by RIA. Follicular fluid samples were obtained from women with normal menstrual cycles (Normal) and women diagnosed with ovulatory or anovulatory PCOS. Each bar represents the average concentration \pm SEM of the steroids measured by specific RIAs (see “Experimental Procedures, 6.2.6) in the study groups. The summary of the RIA data is shown in Table 6. 4. Significant differences in levels of steroid when compared with controls are indicated by a ($P < 0.01$) and b ($P < 0.05$). c represents significantly increased levels of androstenedione in follicular fluids from ovulatory PCOS ovaries compared to anovulatory ovaries. n represents the number of follicular fluid samples assayed in each RIA.

samples, attempts were made to isolate the extracted steroids prior to the actual measurement. However the systems used to separate the steroids, i.e., HPLC and TLC

where problematic in that the recovery of known amounts of unlabeled or radiolabeled 5 α -A from procedures incorporating such steps was irreproducible (results not shown). Therefore, the assay that was developed measured 5 α -A as well as androsterone and androstenedione, simultaneously. Although such an assay was limited in measuring the specific levels of 5 α -reduced androgens, it was useful in studying the patterns of the steroids' levels in biological samples.

The levels of 17KAs measured in follicular fluids from normal, ovulatory and anovulatory PCOS ovaries were 364.5 ± 46.3 , 828.7 ± 168.1 , and 618.9 ± 127.9 nM (mean \pm SEM), respectively (Table 6.5). Given that the 17KA RIA measured the three steroids collectively, it is not possible to make specific inferences of the individual steroids directly using the data obtained by the assay. In addition, androstenedione is the most abundant androgen in the ovary and it is likely that the steroid levels detected by the 17KA RIA largely reflected the concentrations of androstenedione in the follicular fluids. However, in spite of this limitation, it was shown that the levels of the 17KAs measured collectively were approximately 2 - 3 times those of the androstenedione levels that were measured using a specific assay (Figs. 6.7A & B), suggesting that the 17KA RIA measured other 17KAs in addition to androstenedione. It was also shown that the levels of the 17KAs measured collectively were higher in follicular fluids from women with PCOS regardless of ovulatory status, when compared with samples from normal subjects, and that the elevated androgen levels were significantly higher in follicular fluids from anovulatory PCOS compared to the controls ($P < 0.01$; Figs. 6.7A & B).

To estimate the levels of 5 α -17KAs in the follicular fluids, the mean androstenedione value for each group was subtracted from the corresponding mean 17KA value. The concentrations of 5 α -17KAs obtained for follicular fluids of women in the follicular phase of normal menstrual cycles and women diagnosed with ovulatory and anovulatory PCOS were 220.7 ± 30.9 , 564.4 ± 134.0 and 435.1 ± 101 nM, respectively. It is important to note that the 5 α -17KA values obtained were only an approximation due to variable cross-reaction of the individual steroids quantified with the antiserum used. Still, the levels of 5 α -17KAs were again shown to be raised in polycystic ovaries compared to normal (Fig. 6.8). The levels in follicular fluids from

ovulatory polycystic ovaries were approximately 2.5 times those in normal ovaries while levels in anovulatory polycystic ovaries approximated twice the levels in normal follicular fluids. However, there was no significant difference between the levels of 5 α -17KAs in follicular fluids in ovulatory or anovulatory PCOS.

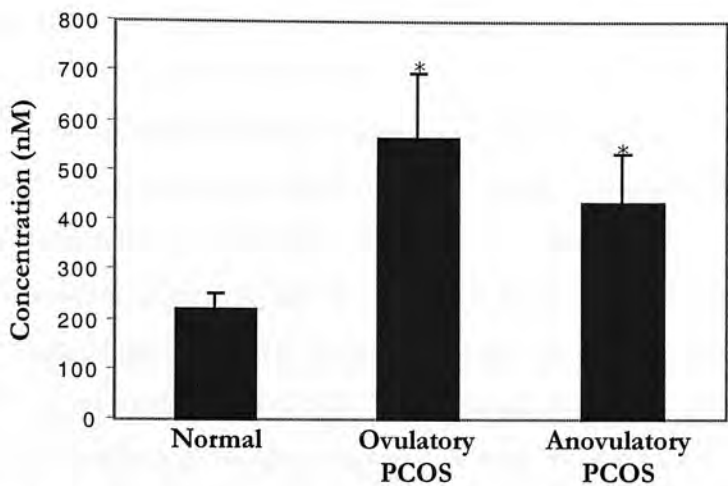


FIGURE 6.8 Levels of 5 α 17KAs in follicular fluids. The levels of 5 α 17KAs in follicular fluids of women in the follicular phase of normal menstrual cycles (normal) and women diagnosed with ovulatory and anovulatory PCOS were estimated by subtraction of the specific androstenedione concentrations from the corresponding total 17KA values. Each bar represents the mean 5 α 17KA concentration \pm SEM (nM). Asterisk (*) denote markedly increased levels of 5 α 17KA compared to normal.

The observed hyperandrogenemia supports earlier works where elevated levels of androgens were reported in women with PCOS (Speroff *et al.*, 1983; Gilling-Smith *et al.*, 1994; Agarwal *et al.*, 1996; Legro *et al.*, 1998; Dewailly, 1999; Nelson *et al.*, 1999; Dunaif & Thomas, 2001)). The estimates correlate with the 3-fold increase in levels of 5 α -A in follicular fluid from polycystic ovaries compared with ovaries with 6-8 mm cohort follicles from regularly menstruating women (Agarwal *et al.*, 1996). Raised androstendione levels have been illustrated *in vitro* using thecal cells from PCOS subjects (Gilling-Smith *et al.*, 1994). It is thought the characteristic hyperandrogenemia in PCOS is due to increased androgen production within the ovaries (Gilling-Smith *et al.*, 1997). Specifically, it has been suggested that the production of 5 α -reduced androgens occurs as a result of the upregulation of the 5 α -R1 as indicated by Jakimiuk *et al.*, (1999) and Chin *et al.*, (2000).

In contrast the levels of oestradiol were significantly low in follicular fluids of both PCOS subtypes, i.e., ovulatory ($P < 0.05$) and anovulatory PCOS ($P < 0.01$; Fig. 6.7C). It is worth noting that oestradiol production was also significantly lower in anovulatory PCOS compared to ovulatory PCOS suggesting that the anovulation in these subjects could be explained in part by lack of adequate levels of oestradiol, which is necessary to attain ovulation. Collectively, the findings suggest that high levels of the non-aromatisable 5α -17KAs co-exist with decreased oestradiol production in PCOS.

The data suggests that the enzymes responsible for 5α -A, androstenedione and androsterone synthesis are upregulated in PCOS while aromatase is inhibited. Infact, oestrogen production was inhibited by 5α -A in human granulosa cells in a dose-dependent manner suggesting that the increased concentrations of 5α -A (as in PCOS follicular fluid) inhibit aromatase action (Agarwal *et al.*, 1996). Similar inhibition was also shown in rat models (Hillier *et al.*, 1980a; Ebong & Peddie, 1990). An appreciable fraction of the androgenic output of ovaries of women with PCOS ovaries constitutes of 5α -reduced androgens. It is known that 5α -reduced androgens are constituents of normal ovaries (Eckstein *et al.*, 1970; McNatty *et al.*, 1979; Gore-Langton & Armstrong, 1988). Although the steroids do not inhibit aromatase action when produced within physiologically normal ranges, they become inhibitory when their production is elevated markedly as can occur in PCOS (Agarwal *et al.*, 1996). Since the follicles in PCOS were shown to be at stages of arrest as opposed to being atretic (Fauser, 1994; Takayama *et al.*, 1996), it is likely that the increased metabolism of androstenedione and testosterone into 5α -reduced androgens contributes to the local ovarian environment that maintains follicles at small undeveloped sizes due to the lack of adequate oestrogen production. The result is a multi-follicular ovarian phenotype that is characteristic of PCOS. The lack of adequate oestrogen levels also perpetuates the inhibition of follicle development by removing the ovarian oestrogenic signal that stimulates and augments FSH secretion from the pituitary. As a result, follicle developments arrests and the development-dependent changes that metabolise androgens into oestrogens are reduced greatly. The outcome is the exaggerated androgenic output that exists alongside reduced oestrogen production in PCOS as illustrated in Fig. 6.7.

Chapter 7. Summary

During reproductive years, the adult ovary is a dynamic organ, which undergoes continuous structural and functional changes that are predominantly controlled by gonadotrophic signals. The main ovarian steroidogenic processes involve conversion of androgenic precursors into oestrogens, mainly oestradiol which is a prerequisite for ovulation (Adashi, 1994; McGee & Hsueh, 2000). Because this function is dependent on the enzymes present in the ovary, understanding the role(s) of ovarian enzymes that metabolise androgens are vital to reproductive function. The focus of the studies described in this manuscript is the 5 α -reductive metabolism of ovarian androgens. The studies were performed in the rat which has also been shown to be a good animal model to study the pathophysiology of PCOS (Mahesh *et al.*, 1987).

Investigations of the metabolism of androstenedione, the most abundant androgen in the ovary were performed in cell culture systems of granulosa and thecal cells isolated from immature Wistar rats stimulated *in vivo* with gonadotrophins (Chapter 3). As expected, oestradiol production was evident in granulosa cell cultures because of the expression of the enzyme aromatase by these cell types (as shown in Chapters 4 & 5). In reference to previous reports of the conversion of androstenedione into 5 α -A by granulosa cells *in vitro* (Hillier *et al.*, 1980a) and knowing that 5 α -reduction is an alternate pathway to aromatisation during ovarian androgen metabolism (Aono *et al.*, 1981; Payne *et al.*, 1992), it had been expected that the immediate 5 α -reduced metabolite of androstenedione, 5 α -A could be detected and measured in cell cultures as part of studies investigating the role of 5 α -reduced androgens in the ovary. However, 5 α -A was not detected in granulosa cell cultures even though oestradiol and testosterone were detected in the same cultures (Chapter 3). Thus, the main enzyme in the granulosa cells in immature rat ovary was shown to be 17 β -HSD, which is expressed constitutively in these cells (Bogovich & Richards, 1984). Disappointingly, despite concerted efforts, the thecal cell cultures failed to show any metabolism in any of the experimental cultures.

In view of the findings shown in Chapter 5, it is now clear why 5 α -reductive metabolism was absent from granulosa cells. The rat ovary expressed the isozyme, 5 α -

R1 which was localised specifically to the theca and interstitial cells where it was up- and down-regulated by LH and FSH, respectively (Chapters 4 & 5). Notably, the enzyme was absent in the granulosa and stromal compartments of the ovary (Chapter 5). It was also illustrated that 5 α -R1 is expressed abundantly in ovaries of immature rats and barely detectable in adult ovaries. If this finding holds true for other species, it suggests that the inhibitory action of 5 α -reduced androgens on oestrogen synthesis (as suggested in Chapter 5) is principally a phenomenon of immature, prepubertal ovaries. It is possible that the 5 α -reduced androgens formed in immature animals are part of the intraovarian cues which ensure that follicular development, particularly granulosa cell proliferation and the ensuing oestrogen production are suppressed in immature animals. They might also have an additional role as steroid intermediates in degradative pathways, which ensure removal of steroids that would otherwise be used as substrates for precocious prepubertal synthesis of oestrogens. Infact, the lack of 5 α -R has been postulated to be fatal in fetal animals due to oestrogen excess (Mahendroo *et al.*, 1997). Therefore, the enzyme probably has a different function in normal adulthood where it might be more important in physiological responses related to pregnancy such as parturition as suggested by (Mahendroo *et al.*, 1999). However, it can still be an important catalyst of 5 α -reduced androgens if high levels of substrate are present. It is very likely that in a condition like PCOS where exaggerated androgen production provides excess substrate, 5 α -R1 may convert androstenedione opportunistically into 5 α -A and subsequently into andosterone, resulting in an intraovarian microenvironment that suppresses aromatase action locally. In such situations, 5 α -reduced androgens act as paracrine modulators of oestrogen production. Further studies clarifying the localisation of ovarian 5 α -R in other species, particularly man are important.

Analyses of RIAs performed on follicular fluids from normal and polycystic women showed evidently that PCOS is a condition of elevated levels of androgens (Chapter 6, Fig. 6.7A & B). In contrast, the levels of oestradiol were decreased significantly in PCOS. Given that PCOS is a multi-factorial disorder, the studies described in this manuscript attempted to analyse the levels of 5 α -reduced androgens in follicular fluid because they are inhibitors of oestrogen production. Hence, they are potential local enhancers of the pathophysiology of PCOS. Even though the non-specificity of the antiserum used to detect the 5 α -reduced androgens rendered the assay

incapable of quantifying specific steroids, the comparison of the levels of 5α 17KA, androstenedione and oestradiol in the follicular fluids illustrated the elevated androgens in PCOS, particularly the ovulatory PCOS. Possibly, ovarian function in anovulatory PCOS, for example, aromatase activity is greatly impaired such that the conversion of androgens into oestrogens is hindered even in the presence of high levels of androgenic substrate. Since 5α -reduced androgens inhibit aromatase locally, it is likely that unknown cellular transcription factors are also involved in effecting this response. Investigation of these sub-cellular modulators could provide clues to specific therapies targeting the action of 5α -R1 in conditions of hyperandrogenemia.

Although the described studies investigating 5α -R activity were done in the rat to address the physiology of a human condition, PCOS, the findings are still relevant because the preovulatory follicular developments within the ovaries of both species are similar. Although man is monoovulatory while the rat is multiovulatory, the regulation of preovulatory development in both species is mainly by gonadotrophins. The sequence of preovulatory follicular development is initiated by FSH stimulation and ends with LH action during ovulation. Thus, studies of the gonadotrophic regulation of 5α -R1 activity in the rat model are relevant to the regulation of 5α -R1 in the human ovary. In addition, the cheaper upkeep of rats as animal models compared to monoovulatory animals such as sheep also makes the rat a good experimental model to study preovulatory development.

Together, all the data indicate that under gonadotrophic control, ovarian androgen metabolism via aromatisation and 5α -reductive pathways occurs in a synchronised fashion related to follicle development. Because of the polarised localisation of aromatisation and 5α -reduction in granulosa and theca/interstitial cells, respectively, the two processes are distinct ovarian steroidogenic processes that occur concurrently to create an intraovarian environment that enhances the follicular changes at each stage of development. Thus, nature has ensured that the release of eggs capable of fertilisation occurs when the female is physiologically mature to carry a conception to term so that the perpetuation of its species is assured.

7.1. Future Work

As discussed earlier (Chapter 2), the roles of 5 α -reduced androgens in ovarian physiology are still unclear. This has been largely because these steroids are quantitatively minor in female reproductive endocrinology. In addition, recent progress in research on 5 α -R and its metabolites in women has been in dermatology (Thiboutot *et al.*, 2000) rather than ovarian physiology because the stimulation of acne, hirsutism and other masculinising symptoms are a major concern of the cosmetic industry. However, the finding of increased expression of ovarian 5 α -R1 (Chapters 4 & 5) during the periovulatory window leads to investigations of the role of 5 α -reduced steroids during this phase of ovarian function.

Whereas 5 α -reduced progestanes were identified as excretory products of progesterone (Eckstein *et al.*, 1970; Lerner & Eckstein, 1976), it is still unclear whether that is the only role of 5 α -R1 during ovulation. Investigations are necessary to clarify how 5 α -reduced metabolism affects ovulation. The strategies adopted could be use of *in vivo* studies in animal models treated with 5 α -R inhibitors. The selected animals must have normal ovarian cycles. By targeting treatments for just before ovulation, the role of 5 α -R1 on ovulation can be studied by immunocytochemical techniques highlighting the morphological developments within the ovary. Gene expression techniques can also be performed on ovarian tissues to study the expression of 5 α -R1 during ovulation and early luteal phase.

Alongside investigations of 5 α -R, it is important to identify and measure the particular ovarian 5 α -reduced steroids synthesised during ovulation. The levels of steroids could indicate the physiologically important 5 α -reduced steroids during this stage. Ideally, follicular fluid samples would indicate what is occurring locally in the ovary, but these samples are not easily collected. It is easier to collect urine samples, but this results in measurement of metabolites of the 5 α -reduced steroids and may confound investigations of the actual ovarian steroids produced. Measurement of the steroids could also be done on blood samples. Because the less studied 5 α -reduced steroids such as 5 α -A may not have established assays for their measurement, well-characterised antisera should be developed to ensure their accurate measurement. In the absence of such antisera, inclusion of separation techniques in the assay protocols

may improve the quantification studies. The establishment of a standardised 5 α -A RIA would be essential to these studies and could be adapted to measure specifically the levels of the steroid in various biological fluids. Aside the obvious need in reproductive endocrinology, the assay would also be used in dermatological investigations.

The studies of the 5 α -reductive effect on ovulation are important because they can broaden the scope within which contraceptives are modified. And should regulation of progesterone metabolism be indicated, such studies may warrant further research on the function of 5 α -R in pregnancy and the uterus. Infact a physiological role for 5 α -R in pregnancy has been suggested (Mahendroo *et al.*, 1999).

The finding of the 5 α -R1 gene in rat thecal/interstitial cells (Chapter 5) indicated the need to clarify the cellular localisation of the enzyme in other species. It is particularly important to investigate this in women since the enzyme contributes to the pathophysiology of PCOS which is a major cause of anovulatory infertility. With the development of therapeutic drugs that target specific enzymatic processes, such studies would highlight the tissues requiring special study during investigations monitoring the effects of the drugs.

Although the research described in this thesis focussed on female reproductive endocrinology, it is worthwhile to note that 5 α -R is crucial in male physiology. Infact it is essential for the normal development of a male phenotype (Andersson *et al.*, 1991). Hence, studies of the regulation and localisation of 5 α -R action in females might allude to the role of the enzyme in males.

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